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THE EFFECT OF METHYLTHIOURACIL ON THE OXYGEN CONSUMPTION OF THE THYROID, LIVER AND KIDNEYS

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AN ABUNDANT literature has made its appearance as a result of the observations that the sulphonamides, thiocarbamides, and thiouracils provoked hyperplasia of the thyroid (Astwood, 1943, 1944; Kennedy, 1942; Mackenzie and Mackenzie, 1943, 1944; Mackenzie, Mackenzie and McCollum, 1941; Richter and Clisby, 1942), and that the basal metabolism falls in proportion as the hyperplasia increases (Astwood, Sullivan, Bissell and Tyslowitz, 1943). Investigations have been chiefly directed on analyzing more in detail the mechanism of the effects of these substances. The hyperplasia of the thyroid proved to be conditioned by an increased excretion of thyrotropin, which in its turn was due to a decrease in the formation of the thyroid hormone, whereby the checking effect of the hormone on the anterior lobe of the hypophysis declined (Astwood and Bissell, 1944; Astwood, Sullivan, Bissell and Tyslowitz, 1943; Higgens and Ingle, 1946).

Various methods have been employed to elucidate how these substances influence the synthesis of the thyroid hormone. In *in vitro* experiments, by adding thiouracil to the substrate, it was possible to show in the thyroid section that the peroxidase reaction of the follicle cells was checked, while the cytochromoxidase reaction was not affected (Dempsy, 1944). This observation would seem to argue in favour of the peroxidase being necessary for the synthesis of thyroxin, and indicate that the effect of the antithyroid substances is a checking of the effect of this enzyme.

However, it is in the first place by the use of radio-active iodine in experiments both *in vivo* and *in vitro* that attempts have been made

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to reveal the mode of action of these substances. In the case of *in vivo* experiments, it has been proved that the capacity of the thyroid to take up I is greatly reduced after thiouracil treatment (Franklin, Chikoff, and Lerner, 1944; Keston, Goldsmith, Gordon and Charipper, 1944; Larsson, Kesting, Peacock and Rawson, 1945; Rawson, Tannheimer, and Peacock, 1944). According to others the I-uptake is not checked, but the thyroid cells themselves are attacked, which leads to a decreased synthesis of the thyroid hormone (Baumann, Metzger and Marine, 1944). The amounts of diiodotyrosine and thyroxin decrease after medication with antithyroideal substances (Franklin, Chaikoff and Lerner, 1944). In *in vitro* experiments the storage of I was not checked, but its entry into diiodotyrosine and thyroxin was checked (Franklin, Chaikoff, and Lerner, 1944; Keston, Goldsmith, Gordon and Charipper, 1944; Lerner and Chaikoff, 1945). In any case these experiments seem to have shown that the synthesis of the thyroid hormone is seriously interfered with. On the other hand, opinions appear to differ as to whether there is a disturbance in some enzyme system, or whether it is a question of an injury to the follicle cell itself.

With the help of the Warburg method, attempts have been made to analyse which enzyme system or systems are checked. Thus, in *in vitro* experiments, thiouracil in a 0.002 M solution is said to check cytochromoxidase (Paschkis, Cantarow, Rakoff and Tillson, 1945). On the other hand, others consider that this enzyme system is not affected (Lerner, and Chaikoff, 1945; McShan, Meyer and Johanson, 1946).

Jandorf and Williams (1944) showed that the oxygen consumption of the thyroid increases greatly in animals which have been treated with thiouracil for 10–20 days. On the other hand, thiourea added *in vitro* does not have any effect on the oxygen consumption of the thyroid section. The different results as between *in vivo* and *in vitro* experiments are explained by the fact that the cell volume is considerably larger in the former experiments and conditions the increased consumption of oxygen (Lerner and Chaikoff, 1945).

Certain clinical observations, such as blood changes, exanthema, "drug fever," etc. suggest that the antithyroid substances have not a selective effect on the thyroid. Such toxic reactions have also been observed in animals (Mackenzie and Mackenzie, 1943; Meyer, Collins and Marine, 1944). There is experimental support for the suggestion that antithyroideal substances also affect other organs than the thyroid. Thus *in vitro* experiments with thiouracil in 100 mg.% concentration the oxygen uptake of rabbit bone-marrow is checked (Warren, 1945), while, on the other hand, the oxygen consumption of liver from animals which had been treated for longer or shorter periods with thiouracil, was not changed (Jandorf and Williams, 1944).

A considerable period (20 days) of administration of thiouracil in

the food reduced the activity in homogenized liver of both succinoxidase and cytochromoxidase. The cause of this was the reduced synthesis of the thyroid hormone. If thyrotropic hormone, dried thyroid, or thyroxin are administered for 2 weeks a definite increase in the activity of the liver enzyme results. In experiments with liver from both thyroidectomized and thiouracil-treated rats the same activity in the enzyme system in question resulted in both cases, which would suggest that thiouracil does not affect organs other than the thyroid (Tripton and Nixon, 1946). The question whether the antithyroid substances only attack certain enzyme systems in the thyroid which are necessary for the synthesis of thyroxin, or whether they have a more general point of attack is thus still uncertain.

Only one work has dealt with the changes in the oxygen consumption of the thyroid after treatment with thiouracil (Jandorf and Williams, 1944), and the conclusions are based on very few experiments. It therefore appeared to us of interest to carry out such an investigation on a large animal material, and further to attempt to show whether thiouracil has a more general point of attack or only affects the tissue of the thyroid.

MATERIAL AND METHODS

For the present work white rats, all males, were employed. The animals all received the same food. The drinking water consisted of a 0.1% methylthiouracil solution, to which the animals had free and abundant access, and which was renewed every day. The animals were killed after having been on the diet and drinking water indicated here from 1 to 67 days (see table 1). Immediately after the animal had been decapitated a piece of liver, kidney, and left thyroid lobe were excised for determinations of the QO_2 according to Warburg, With regard to the method, see Borell (1945); Holmgren and Naumann (1947).

In some cases we determined the oxygen consumption of liver and kidney from thyroidectomized rats. The animals were given methylthiouracil in the drinking water about 10 days after the operation, when they had recovered from the operation.

The right lobe of the thyroid was always fixed in Susa for 24 hours and subsequently embedded in paraffin in the usual manner.

From three symetrically chosen heights within the lobe of the gland sections 5μ thick were taken and stained with hematoxylin-eosin.

RESULTS

As appears from table 1 and diagram 1, the oxygen consumption for the thyroid is relatively high in the control animals—QO_2 of -7.6. In all probability this is due to the fact that normally the thyroid of rats is active, with high follicle epithelium and but little colloid. Thyroid from guinea-pigs of the same size give QO_2 values of only -3.7 (Borell, 1945) which tallies well with the inactive picture shown throughout by these glands. Even these figures show that

glandular activity and QO_2 are interdependent, a circumstance that appears clearly from Borell's work on cell height and oxygen consumption in cases of treatment with increasing doses of thyrotropine.

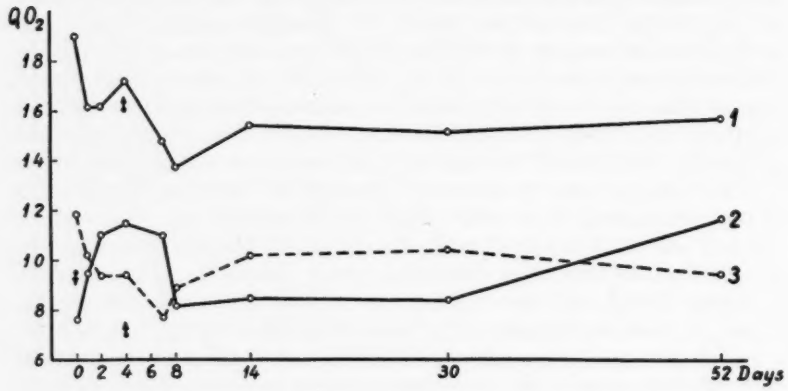

DIAGRAM 1

The oxygen consumption of thyroid, liver and kidney.
1 = Kidney
2 = Thyroid
3 = Liver
♂ = Liver and kidney from thryoidectomized animals and treated with methylthiouracil four days.
♀ = Liver from only thyroidectomized animals.

TABLE 1. THE QO_2 IN THE THYROID, LIVER AND KIDNEY FROM RATS TREATED WITH METHYLTHIOURACIL

Number of days treated with methylthiouracil	QO_2		
	Thyroid	Liver	Kidney
Controls	− 7.6 $n=10$	−11.8 $n=22$	−19.0 $n=9$
1 day	− 9.5 $n=7$	−10.2 $n-7$	−16.1 $n=6$
2 days	−11,0 $n=6$	− 9,4 $n=6$	−16,2 $n=6$
4 days	−11,4 $n=8$	− 9,4 $n=6$	−17,2 $n=8$
7 days	−11,0 $n=6$	− 7,7 $n=6$	−14,8 $n=6$
8 days	− 8,1 $n=6$	− 8,9 $n=6$	−13,7 $n=6$
14 days	− 8,5 $n=8$	−10,2 $n=8$	−15,4 $n=5$
30 days	− 8,4 $n=6$	−10,5 $n=5$	−15,1 $n=4$
67 days	−11,7 $n=12$	− 9,4 $n=12$	−15,7 $n=12$

Even in animals which had drunk water containing methylthiouracil for only 24 hours the QO_2 in the thyroid increased to −9.5. The difference between this value and that exhibited by the control animals is not significant; the great dispersion within the respective groups is remarkable. In the case of the rats which received methylthiouracil the cause must be that the dosage naturally varied from animal to animal, all depending on the amount they drank during the first day of the experiment. Within the control group also the disper-

sion is great, which is explained by the fact that the activity picture of the thyroid in rats varies greatly from case to case.

In rats which received methylthiouracil for 2 days there is a further increase in the QO_2 to -11.0. The difference from the value for the control animals is now statistically probable, with a t-value of 2.86 and $p < 0.02$. On the other hand, the difference between the control animals and the animals which had received methylthiouracil for 4 days is significant. As appears from table 1, the QO_2 value is about -11 for animals which had consumed thiouracil for 4 to 7 days, but after 7 days a very marked fall in the QO_2 takes place down to values of -8. The fall is statistically verified, with a t-value of 3.30 and $p < 0.01$ (see table 2). Finally, we found the highest QO_2 value for the oxygen consumption of the thyroid from rats which had received

TABLE 2. STATISTICAL CALCULATIONS OF THE DIFFERENCE BETWEEN THE MEAN VALUES FOR THE QO_2 IN THE THYROID, LIVER AND KIDNEY

Groups between which the difference was investigated		t	p	Organ
A.	B.			
Normal animals	1-day animals	1,84	<0,1	
Normal animals	2-day animals	2,86	<0,02	
Normal animals	4-day animals	4,01	<0,01	Thyroid
4-day animals	8-day animals	3,30	<0,01	
30-day animals	67-day animals	2,70	<0,02	
Normal animals	1-day animals	2,08	<0,05	
Normal animals	2-day animals	3,42	<0,01	
Normal animals	4-day animals	3,40	<0,01	
7-day animals	8-day animals	1,25	<0,3	Liver
7-day animals	14-day animals	2,17	<0,1	
7-day animals	30-day animals	3,06	<0,02	
30-day animals	67-day animals	0,66	<0,6	
Normal animals	1-day animals	1,59	<0,2	
Normal animals	2-day animals	2,00	<0,1	
Notmal animals	4-day animals	1,18	<0,3	
Normal animals	7-day animals	4,53	<0,001	
Normal animals	8-day animals	3,94	<0,01	Kidney
8-day animals	67-day animals	0,84	<0,5	

methylthiouracil for 67 days. The difference (-3.3) between this value and the value for the 30-day animals (8.4) is statistically probable with $p < 0.03$.

It is of special interest that the liver and kidney respiration changes during methylthiouracil medication in quite a different manner from that of the thyroid. As appears from table 1 and diagram 1, for these two organs the QO_2 value falls already during the first 24 hours of the treatment, and the reduction continues, successively on the whole, until minimum values are reached after 7–8 days. The liver respiration of the control animals (-11.8), which tallies well with other observations (Holmgren and Naumann, 1947), differs significantly from the QO_2 for liver from rats killed after 2 days' treatment

(−9.4). The difference is −2.4 and the t-value 3.42, with a $p < 0.01$.

On the other hand, a statistically verified difference in kidney respiration as between the control animals and those treated with methylthiouracil does not show itself until after 7 days' treatment, with a t-value of 4.53 and $p < 0.001$. If the treatment is continued for a considerable period, 30 to 67 days, the QO_2 for liver and kidney shows no further change.

It is obvious that the kidney sections within the respective groups give very varying values for QO_2, and the dispersion is remarkably great.

A group of 12 rats were thyroidectomized, and 5 of them were put on water containing methylthiouracil about 10 days after the operation and kept on this fluid for 4 days, after which they were killed, and the oxygen consumption determined on liver and kidney sections. These experiments were made to ascertain whether methylthiouracil possibly affected other organs than the thyroid. The QO_2 in rats which were only thyroidectomized was −9.2, which is less than that of the normal animals. The QO_2 in the liver of rats which had been operated on and received methylthiouracil was considerably lower, however, amounting to −6.9. The difference between the liver respiration in the respective groups is statistically probable, with t-values of 3.11 and $p < 0.02$. The corresponding values for kidney sections were −16.0 and −16.3 respectively. The individual values in the different experiments appear from table 3.

DISCUSSION

A more detailed analysis of the results of the experiments renders possible a discussion of the mode of action of methylthiouracil.

The actual synthesis of thyroxin is associated with oxidative processes, chiefly in the conversion of iodide to free iodine. However, according to a number of investigations with radioactive iodine, methylthiouracil appears to check just that process.

Thus the increase which we found in the oxygen consumption of the thyroid gland may not be due to an increased formation of the hormones of the gland. Just by investigating thyroids treated with methylthiouracil a possibility is presented of a closer study of the changes conditioned by processes in the gland other than those directly associated with hormone function.

The circumstance that there is an increase in the oxygen consumption in the thyroid, but a reduction in the consumption of the liver and kidney, does not exclude the possibility that it is the same enzyme system in the different organs that is affected, for there is much which indicates that the increase in the oxygen consumption of the thyroid after methylthiouracil is chiefly conditioned by an increase in the size and number of the follicle cells of the thyroid. If the QO_2 is calculated, and not the total oxygen consumption of

the gland, the effect of an increased volume of the gland is avoided. It is then probably only the cell increase itself which is registered. Experiments which have been carried out earlier also speak in favor of the correctness of this argument, for, in the case of injections of small amounts of thyrotropin, a great rise in QO_2 was met with during the time the follicle cells increased greatly in size, but, in spite of further injections over a couple of days, no changes could be proved either in cell height or QO_2 (Borell, 1945). Other experiments also indicate the correctness of this. By determining the number of mitoses in the thyroid after treatment with methylthiouracil for varying numbers of days, it has been possible to show that on the whole the changes in the number of mitoses follow the changes we found in the QO_2 (Paschkis, Cantarow, Rakoff and Rothenberg, 1945; Rerabek, 1946). That the increase in the QO_2 of the thyroid is not a direct effect of the methylthiouracil appears from the fact that the QO_2 in the thyroid decreases, in spite of continued supplies of methylthiouracil.

It appears to be of interest that the histological picture of the thyroid is still active after more than 60 days' methylthiouracil treatment, for, according to a number of authors, repeated injections of thyrotropin provoke a return to inactivity, owing to the formation of an antithyrotrophic substance (Anderson and Collip, 1934; Collip and Anderson, 1935; Eitel and Loeser, 1935). Others, however, state that the cause is the development of antibodies against the unspecific protein substances which accompany the preparation extracted from the anterior lobe (Katzman, Wade and Doisy, 1937; Werner, 1936). Our experiments support this opinion, as in the rats treated with methylthiouracil the content of thyrotropin increases and maintains a histological active thyroid gland. In these cases naturally no unspecific protein is met with.

If the synthesis of the thyroid hormone is entirely stopped by methylthiouracil, it should be expected that, on the whole, the oxygen consumption of the liver would be the same in thyroidectomized animals as in those treated with methylthiouracil. The results show that, on the whole, the same values are obtained under both sets of experimental conditions. It is possible that the oxygen consumption is somewhat higher in the treated animals. An explanation of this may be that the synthesis of thyroid hormone is not entirely abolished. Further, it is known that the effects of thyroxin do not disappear rapidly but persist for a very long time. Thus it should still be possible to trace the effect after about a month. Others have found no change in the oxygen consumption of the liver after methylthiouracil (Jandorf and Williams, 1954). Thuss we cannot verify these results. The reason must be that those authors used small series, and their values shows great dispersion.

Results of the experiments in which thyroidectomized animals were treated with methylthiouracil also appear to be of interest (table

3). In this case the respiration of the liver was considerably lower than that of the control animals, which had only been thyroidectomized. This definitely indicates that methylthiouracil also attacks other organs than the thyroid. In similar experiments certain enzyme systems taking part in the oxidative processes have been investigated, but no difference could be proved as between the experimental series (Tripton and Nixon, 1946). To a certain extent this conflicts with our results, but it may be explained by the fact that these authors only determined the content of individual enzyme systems, while in our experiments we measured the total consumption of oxygen.

TABLE 3. THE QO_2 IN KIDNEY AND LIVER SECTIONS FROM THYROIDECTOMIZED ANIMALS

Liver		Kidney	
Controls	Treated with methylthiouracil	Controls	Treated with methylthiouracil
10,2	5,6	11,1	13,9
7,4	6,9	16,8	19,2
11,1	7,1	18,5	15,9
7,2	7,9	14,4	16,0
9,8	7,1	15,7	16,3
10,2		19,7	
8,8		15,9	
9,24	6,92	16,01	16,26

Finally, with regard to the respiration experiments with the kidney, on the whole the QO_2 in the kidney changed analogously with that of the liver. Since the dispersion of the individual values was very great, statistically verified differences from normal values were not obtained until after 7 days' treatment with methylthiouracil. After thyroidectomy the same oxygen consumption in the kidney is found, both with and without methylthiouracil. The individual values exhibit very great dispersion (table 3). The cause of this is probably that tangential sections of the kidneys were taken, in which case the quantity of medulla and cortex varies greatly from section to section. These two parts of the kidney have different QO_2 values, and therefore the variations in their amounts probably play an important role.

SUMMARY

In the present work the effect of methylthiouracil on the consumption of oxygen in the thyroid, liver and kidney, has been studied. Further, the effect of this substance on thyroidectomized animals has been investigated. On the whole the results were as follows:

The oxygen consumption of the thyroid increases rapidly after the administration of this substance in the rats' drinking-water. The increased consumption is probably due to growth of the follicle cells of the thyroid. In spite of further supplies, the respiration falls.

The livers from thyroidectomized animals which have been treated with methylthiouracil show a lower oxygen consumption than do those of the control animals. This argues definitely in favor of the assumption that the methylthiouracil had a general effect and did not selectively attack the thyroid.

The oxygen consumption of the liver and kidney falls relatively soon after methylthiouracil treatment is begun. The cause of this appears to be a decreased synthesis of thyroid hormone. Further, there is a clear—at least as far as the liver is concerned—direct effect of methylthiouracil.

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HYPERVOLEMIA IN MICE BEARING GRANULOSA CELL GROWTHS; TIME OF ONSET AND SOME ASSOCIATED PHYSIOLOGICAL AND CHEMICAL CHANGES[1]

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IT WAS NOTED recently that mice bearing subcutaneous growths of transplanted granulosa cells have enormously congested livers and adrenals (Furth and Boon, 1945) and an associated hypervolemia with 2 to 3 times normal values (Furth and Sobel, 1947). The hypervolemia is present in mice bearing transplanted granulosa cell tumors originally induced by x-rays (Furth and Sobel, 1946) or produced by intrasplenic grafts of ovarian fragments into gonadectomized mice (Furth and Sobel, 1947). It was suggested that this increase in blood volume was produced, directly or indirectly, by a hypothetical substance elaborated by the neoplastic granulosa cell. The physiological and chemical studies reported here were undertaken to learn more about the nature of this hypervolemia.

MATERIALS AND METHODS

The origin of the transplantable granulosa cell tumors used in this experiment has already been described (Furth and Sobel, 1946; 1947). Strain V, an x-ray induced tumor, was in its 8th and 9th passage with one exception: the tumor carried by mouse 1483 was in its 3rd passage and the similar strain B2 in its 3rd and 4th passage. A few tumors of Strains I and III were also studied. The tumors examined have been growing faster than in the earlier passages and this may account for the decreased degree of associated hypervolemia.

In order to determine the blood volume at repeated intervals the technic previously used (Furth and Sobel, 1946) has been modified so that samples could be taken without killing the mouse. 0.20 cc. of Evans Blue solution (0.15–0.30 per cent in saline) was injected into a tail vein of a mouse that had been anesthetised with nembutal. The jugular vein was exposed through a small incision and 0.1 or 0.2 cc. of blood was drawn into a small syringe, that had been rinsed with heparin solution, using a 27 gauge needle. Bleeding was stopped by pressure and the wound was sutured.

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[2] Research fellow of the David, Josephine and Winfield Baird Foundation.

The repeated determinations could be carried out one week apart for it was found that the dye was removed from the blood stream by this time. This procedure has been repeated up to 5 times on the same mouse. It was important to reduce trauma and infection to a minimum since scarring made subsequent bleedings more difficult.

To 0.10 cc. of the blood sample was added 0.90 cc. of isotonic sodium citrate solution. After centrifugation the concentration of the dye in the supernatant was determined at 575 mμ in the Beckman spectrophotometer[3] using microcells with approximately 0.7 cc. capacity described by Lowry and Bessey (1946).[4]

When the thiocyanate space was measured simultaneously with blood volume (Gregersen and Stewart, 1939), 1.0, 1.5 or 2.0 mg. of sodium thiocyanate in 0.2 cc. of solution was injected into the tail vein of mice 24 minutes before the injection of the dye. The mouse was exsanguinated. 0.1 cc. of plasma was put into 0.65 cc. of distilled water and the proteins were precipitated by 0.25 cc. of 20 per cent trichloracetic acid. To 0.5 cc. of the supernatant was added 0.5 cc. of the ferric nitrate solution and the color was read at 460 mμ in micro cells of the Beckman spectrophotometer. The difference between the thiocyanate space and plasma volume will be referred to as the "extracellular-extravascular space." The total volume of the solutions injected may have a significant influence on the body water of the mouse and the values obtained are regarded as relative.

The total plasma protein and albumin were determined by the method of Greenberg (1929) using the usual constants but smaller quantities of plasma[5] (0.100 cc. for albumin and 0.050 cc. for total protein). The blood urea was determined by the method of Archibald (1944) using 0.10 cc. of blood.

The water tolerance test was performed by administering by stomach tube water equivalent to 5 or 10 per cent of the body weight. When 10 per cent was given, it was administered in two doses at 30 minute intervals. The feces and urine were first expelled by gentle massage and the mouse was placed in a metabolism cage made by suspending a wire support into a 4 inch funnel sealed at the end of the calibrated stem. The urine volume was measured at hourly intervals for 3 hours. The results were expressed as the ratio of the per cent of water recovered after the third hour from the tumor bearing mouse to the per cent recovered from the normal control mouse.

For blood glucose determination by a modification of the Folin-Wu method (1920) 0.02 cc. of blood was obtained from the tail of the heparinized mouse using a special white cell counting pipette. The blood was diluted in the pipette, transferred to 0.70 cc. of water and the proteins were precipitated with 0.10 cc. of tungstic acid. To 0.70 cc. of the supernatant fluid 0.50 cc. of the copper reagent was added and the air was excluded by a glass plug. After the usual heating period the cooled mixture was treated with 2 cc. of a 1:1 mixture of phosphomolybdic acid reagent in glacial acetic acid and the concentration was determined using a visual colorimeter with micro attachments.

When glucose tolerance was determined 0.004 cc. per gm. of mouse of a

[3] We wish to thank Dr. H. L. Barnett for the use of the spectrophotometer.

[4] Manufactured by the Pyrocell Company.

[5] Some of the values were determined through courtesy of Miss E. M. Russ by Miss M. Hekimian.

TABLE 1. REPEATED BLOOD VOLUME DETERMINATIONS ON MICE BEARING GRANULOSA CELL TUMORS

Mouse No.	Strain	Tumor size[1]	Days after inoculation	Weight of mouse	Blood vol. % body weight[2]	Red cells	
						$\times 10^6$ per cu. mm.	$\times 10^6$ per gm.[3]
2134	V	18.3	55	26	10.6	8.2	8.7
		20.3	62	30	9.4	9.0	8.5
		21.6	70	34	16.7	10.6	7.7
2189	V	16.3	30	25	9.6	9.3	8.9
		25.0	40	29	18.4	5.2	9.6
2190	V	14.6	38	24	8.7	9.8	8.5
		20.0	48	25	13.3	6.7	8.8
		20.9	55	26	20.9	6.2	12.9
		21.6	62	26	28.4	3.6	10.2
2191	V	15.0	25	21	9.6	7.2	6.8
		19.0	35	20	21.7	4.0	8.6
2207	V	13.6	55	24	10.4	11.0	11.1
		21.3	63	27	9.9	8.6	8.5
		29.2	77	30	18.5	4.0	7.4
2201	V	26.3	47	28	14.1	7.2	10.1
		33.3	52	26.5	18.1	—	—
2348	V	11.6	153	30	9.5	9.5	9.0
		11.6	160	30	10.6	9.7	10.3
		17.3	174	31	10.6	9.2	9.8
		20.2	184	33	11.2	10.1	11.3
		23.0	198	29.5	17.3	7.2	12.4
2383	V	25.3	60	29.5	15.0	10.4	15.5
		27.3	68	28	15.5	8.9	13.7
2375	V	30.6	60	30	14.0	7.1	10.0
		30.6	68	32	20.0	6.0	12.0
1483	V	11.3	389	34	8.1	9.4	7.6
		13.0	447	36	9.2	9.0	8.3
		14.3	466	35	9.8	7.9	7.8
2135	B1	11.3	63	21	10.9	9.1	9.9
		18.5	87	24	11.0	11.2	12.3
		23.0	93	23	9.4	9.9	9.3
		27.6	103	23	17.1	6.3	9.3
2187	B1	14.6	53	24	8.4	12.1	10.2
		25.6	68	27	23.0	5.6	12.9
2188	B1	10.6	77	21	9.0	10.1	9.0
		13.3	85	23	9.3	11.6	10.8
		20.3	92	24	8.9	9.2	8.0
		24.3	102	26	15.6	4.4	6.8
2345	B2	19.6	72	36	9.2	10.3	10.1
		21.3	79	35	10.2	8.4	8.6
		21.3	86	34	11.4	8.3	9.4
		22.9	93	31.5	12.2	7.3	8.8
2389	B2	10.0	115	29	8.8	9.2	8.0
		11.6	136	29	9.5	9.3	8.0
		21.3	189	31	14.3	7.5	10.7
		23.6	199	32	14.7	5.8	8.3
10 mice, normal					8.6–10.9 (9.9)	7.5–10.4 (8.7)	6.4–11.4 (8.6)

[1] The size of the tumor is arbitrarily given as the average of the three greatest diameters as determined by approximate external measurement.

[2] Based on total weight of mouse.

[3] Blood Volume (cu. mm.) $\times$ R.B.C. in millions / Weight of mouse.

50 per cent glucose solution was injected into the tail vein. Samples of blood were taken before injection and at 15, 45, 75 and 105 minutes after injection. It was usually necessary to inject heparin again before the 4th specimen was taken.

RESULTS

Repeated blood volume determinations. The data presented in Table 1 show that the time of onset of hypervolemia was unpredictable and that the blood volume rose rapidly once the mechanism was set in motion. E.g. in mouse 2191a hypervolemia was present on the 35th day after inoculation with tumor particles while with mouse 2348 carrying the same strain of tumor hypervolemia did not occur until the 198th day. The blood volumes of mice 2134, 2189, 2191, 2135 and 2188 rose from normal values (below 10 per cent of body weight) to values between 15.6% and 21.7% of body weight in 8 to 10 days.

A correlation of blood volume with tumor size as expressed by the average of the three greatest dimensions revealed that there was a minimal tumor size below which hypervolemia did not appear, and that a mouse may have a very large blood volume while carrying a smaller tumor than another with a normal blood volume. E.g. mouse 2191 bearing a tumor of 19.0 mm. had a blood volume of 21.7% of body weight while mouse 2135 had a tumor of 23.0 mm. with a normal blood volume. Once the blood volume began to rise small increases in tumor size were accompanied by great increases in blood volume. E.g. in mouse 2134 the blood volume increased from 9.4% to 16.7% while the tumor grew from 20.3 mm. to 21.6 mm. The blood volume of mouse 2375 increased from 14.0% to 20.0% while the tumor size was not significantly altered. Successive determinations of blood volume of mouse 2190 yielded figures of 8.7%, 13.3%, 20.9% and 28.4% body weight while the tumor size was 14.6 mm., 20.0 mm., 20.9 mm. and 21.6 mm. respectively. Thus it is evident that the blood volume increases rapidly once it begins to rise.

Strain B2 granulosa cell tumors were associated with only slight hypervolemia, strains B1 and V with marked hypervolemia. Cells of all 3 strains secrete estrogens. (Furth and Sobel, 1946, 1947).

Red Blood Count During Hypervolemia: It was reported previously (Furth and Sobel, 1946) that in hypervolemic animals bearing strain V granulosa cell tumors the total number of circulating erythrocytes had increased although the hematocrit values were usually decreased. Mice bearing the tumors had an average of 10.2×10^8 erythrocytes per gram of mouse while normal mice had an average of 5.3×10^8 erythrocytes per gram.[6]

This change appears to have been lost in the course of successive passages of Strain V. In the course of the current studies, the total number of erythrocytes per gram of animal was in the normal range

[6] These values were based on the blood volume as determined by exsanguination—perfusion and are approximately one half of those obtained by the dye technique.

except in mouse 2383 that had a somewhat increased number of red cells. Two mice bearing the B1 tumor (not reported in the table) had 18.2×10^8 and 13.0×10^8 erythrocytes per gram of mouse.

The Extracellular Fluid: The finding of hypervolemia raised the question of the distribution of water in other compartments. By the simultaneous determination of thiocyanate space, blood volume, and hematocrit, the "extracellular-extravascular" space was determined. The fluid in this compartment was appreciably increased in only 3 of

TABLE 2. THIOCYANATE SPACE, BLOOD VOLUME AND "EXTRACELLULAR-EXTRAVASCULAR" FLUID IN MICE BEARING GRANULOSA CELL TUMORS

Mouse No.	Strain	Thiocyanate space % body weight	Blood volume % body weight	"Extracellular-extravascular" fluid % body weight
2343	B2	35.4	12.2	26.8
2326	B2	41.4	12.3	34.5
2409[1]	B2[4]	35.3	12.7	27.4
2348	V	44.3	17.3	35.8
2383	V	41.5	15.5	31.6
2375	V	40.3	20.0	24.7
2491	I	35.3	13.6	26.3
2521	B2[4]	37.5	15.5	27.8
2389	B2	37.5	14.7	27.1
2515	V	37.5	18.4	24.7
Normal Values		28.1–33.5[2]	8.4–10.5[3]	23.5–27.0[3]

[1] Ovarectomized.
[2] 15 determinations.
[3] 12 determinations.
[4] These two mice had splenic grafts of granulosa tumors; all others subcutaneous grafts.

The following determinations were thought to be erroneous and were not included in the table: One "normal" mouse had a thiocyanate space of 37.2% and an "extracellular-extravascular space" of 32.3%. One apparently dehydrated "normal" mouse had a thiocyanate space of 24.5%, a blood volume of 7.7%, and an "extraceullular-extravascular" space of 20.1%.

the 10 mice bearing granulosa cell tumors (Table 2). Mouse 2326 had an "extracellular-extravascular" space of 34.5% while its blood volume was only slightly elevated. On the other hand mouse 2491 had a normal "extracellular-extravascular" space with a blood volume of 20.0% of its body weight. Thus an increase in "extracellular-extravascular" space can occur in mice with granulosa tumors but it does not appear to be primarily related to the hypervolemic process. It is known that estrogens can cause the retention of water (Thorn, Nelson and Thorn, 1938).

Plasma Proteins. The great increase in plasma volume raised the question of its effect on blood constituents. The finding on plasma proteins are presented in Table 3. Of a total of 15 mice bearing granulosa tumors, 4 had subnormal albumin values and one, (2364), had an increased value, the rest were approximately normal. The globulin values were approximately normal in 8 mice, markedly below normal

in 5. Five mice had normal total protein levels. In mice 1414, 1850 and 2146 the exsanguination blood volume was 5 cc. or more, an enormous increase; nevertheless, the albumin levels were normal.

Thus, it is apparent that in nearly all cases this hypervolemia is associated with an increase in the absolute amount of circulating albumin, sufficient to maintain a normal concentration. While there

TABLE 3. PLASMA PROTEIN VALUES OF MICE BEARING GRANULOSA CELL TUMORS

Mouse No.	Strain	Total protein	Albumin	Globulin	Blood obtained[1] cc.	Congestion[2] Liver	Congestion[2] Adrenal
1414	V	4.7	3.7	1.0	6.5	+++	++
1853	V	4.2	3.4	0.8	3.4	++	+
1857	V	4.8	3.6	1.2	4.1	++	+
1925	V	3.2	2.6	0.6	2.0	++	+
1850	V	3.6	3.3	0.3	5.0	+++	++
2105	V	5.0	3.5	1.5	4.8	+	
2153	V	4.9	3.9	1.0	3.0	+++	++
2146	V	3.8	3.3	0.5	5.1	+++	+
2133	B1	4.07	3.29	0.78	1.4	+	
2291	B1	4.63	3.48	1.15	2.8	++	
2132	B1	3.98	3.25	0.73	3.8	+++	
2364	V1	5.33	4.16	1.17	3.7	+++	
2209	B2[3]	4.23	3.14	1.09	1.3	++	
2212	B2[3]	4.12	3.21	0.91	0.7		
2328	B2[3]	4.17	2.89	1.18	0.9		
Average		4.3	3.4	0.9			
Normal plasma[4]							
Extremes		4.4–5.0	3.2–3.7	0.9–1.3	About 1 cc.		
Average		4.7	3.5	1.2			

[1] By cutting jugular vein. These values may be regarded as a crude index of blood volume.
[2] As seen in sections. + = slight; ++ = moderate; +++ = advanced congestion.
[3] These tumors were intrasplenic; all others subcutaneous.
[4] Six determinations on plasma from groups of mice, pooled.

was an increase in the total amount of circulating plasma globulins in several mice, this tendency is not as general as with the plasma albumin.

Blood Urea. It was observed that several mice bearing large subcutaneous granulosa cell tumors had oliguria. Furthermore, it was thought that hypervolemia might possibly result from an imbalance of the renal vasoexcitor and hepatic vasodepressor (Schorr, Zweifach and Furchgott, 1945); therefore determinations of blood urea were made. Table 4 shows values obtained on mice with various degrees and at various stages of hypervolemia and that normal, slightly or greatly elevated levels of blood urea accompanied the hypervolemia. This could mean that the increase in blood urea may be a direct or indirect consequence of the hypervolemic process or occurs independently.

Histology of Hypervolemia: Earlier studies have indicated that hypervolemia is regularly associated with cavernous congestion of

several organs, most consistently the liver and (or) the adrenals (Furth and Sobel, 1946). The degree of cavernous congestion in these organs parallels the degree of hypervolemia and, in absence of blood volume determinations, this can be used as a crude indication of its magnitude. However, the relative degree of congestive changes in these organs varies so greatly as to warrant the assumption that vasodilation in one of these organs alone (e.g., liver or adrenal) is not the prime cause of hypervolemia.

Heart Weight. Blood pressure determinations have not yet been made on mice bearing a granulosa tumor. In order to obtain preliminary information as to a possible coexisting hypertension the hearts of many mice with marked hypervolemia were weighed. There seems to be no clear cut relationship between heart weight and congestive changes as indicated by the following figures:

HEART WEIGHTS OF GRANULOSA CELL TUMOR BEARING MICE
Congestive changes

0	+	++	+++
.52*	.40	.39	.48
	.46	.40	.48
	.60	.49	.56
	.57	.44	.66
		.43	
		.46	
		.43	
Average .52	.51	.44	.55

* Percent of body weight.

The normal heart weight of 10 mice varied between .43 and .54 and averaged .48% of body weight.

The heart weights of some animals were above the average of normal while those of others were below normal. These findings are difficult to explain. Since it requires time for the development of changes in heart size following a rise or drop in blood pressure, these findings do not exclude with certainty hyper- or hypotension in the experimental animals. Determinations of blood pressure in mice before the onset of hypervolemia and during its various phases may yield valuable information as to the nature of hypervolemia.

Further preliminary findings with respect to liver and adrenal function. An investigation was begun on the function of the liver and adrenals in mice bearing granulosa cell tumors. The following observations have been made thus far:

(1) Prothrombin time studies on 9 mice bearing such tumors and with obvious hypervolemia yielded decreased, normal, or increased values.[7] The prothrombin values of 11 normal mice determined by the Herbert two stage method varied between 94% and 106.5%

[7] We are indebted to Dr. W. K. Rieben for these determinations.

averaging 100.18%. Those of 10 mice with granulosa tumors varied between 70% and 124.5%. Two of these mice with subnormal values had liver damage. One had a prothrombin value of 79.5% and a ++ congestion in liver associated with a marked leukemoid reaction; another with a prothrombin value of 70% and an advanced cavernous congested liver (+++) indicative of a marked hypervolemia and numerous infarcts in the liver. One mouse with a greatly elevated prothrombin value (121%) had a marked liver congestion; another

TABLE 4. BLOOD UREA NITROGEN OF MICE BEARING GRANULOSA CELL TUMORS

Mouse No.	Strain	Blood urea nitrogen mg. %	Blood obtained by exsanguination
2120	V	98	3.8 cc.
2123	V	88	1.7 cc.
2121	V	26	4.1 cc.
2119	V	112	3.9 cc.
2153	V	135	3.0 cc.
2116	V	62	5.1 cc.
2118	V	25	3.5 cc.
2199	V	100	3.0 cc.
2206	V	71.5	4.9 cc.
1874	III	100	5.5 cc.
2290	B1	36.8	1.5 cc.
2133	B1	45.0	1.0 cc.
			Blood Volume[1]
2191	V	50	21.7%
2204	V	17.5	8.2%
2190	V	54	28.4%
2201	V	23.5	18.1%
2207	V	27.5	18.5%
2187	B1	75	23.0%
2135	B1	27.5	17.1%
2188	B1	18.5	15.6%
2132	B1	40.5	18.3%
2291	B1	25.0	18.8%
10 mice	Normal	17.5–30	

[1] Per cent body weight. All but last two values (2132, 2291) were obtained by the dye technique; the last two mice were exsanguinated and perfused.

with prothrombin value of 124.5% had a slight liver congestion. In 6 of the 10 mice with granulosa cell tumor tested, the prothrombin values were above the highest normal value of this series (106.5%). Thus hypervolemia itself seems to be associated with an elevated prothrombin level until secondary changes damage the liver and the prothrombin levels drop below normal.

(2) Water tolerance tests were normal in 5 of 6 mice with hypervolemia. The excretion ratios were as follows: 1.15, 0.86, 0.92, 1.30, 0.86 and 0.38.

(3) The blood glucose levels after 6 to 7 hours of starvation of 6 of 12 mice bearing these granulosa tumors but with unknown blood volumes were below 85 mg.%, the lowest value observed in normal

mice during this period. Eight of nine normal mice had blood glucose levels ranging from 105–130 mg.% after 6 to 7 hours starvation. If 105 mg.% is taken as the lower limit of normal then 9 to 12 mice bearing the granulosa cell tumor had low values while 5 to 12 mice bearing other tumors had low values.

(4) Sodium and potassium determinations on the plasma of hypervolemic mice yielded variable results and require further observations.

DISCUSSION

The marked hypervolemia observed occurs only in mice bearing transplanted granulosa cell tumors. This is a unique phenomenon thus far not noted in women with granulosa cell tumors or in mice with spontaneous or induced (non-transplanted) granulosa cell tumors of the ovary. However, in mice at least, the spontaneous or induced ovarian tumor does not attain the bulk of the transplanted granulosa cell tumor. Furthermore no blood volume determinations are available on women or mice with large spontaneous growths of this type.

The phenomenon is dormant for a long time in mice with progressively growing granulosa tumor and then it appears rapidly. This is a puzzling feature the understanding of which may furnish the key to this phenomenon. The ability of the animal to inactivate a hypothetical substance secreted by the tumor or to compensate for it may be overcome by the increasing amounts of this substance.

Relation to estrogens. The neoplastic granulosa cells here described produce estrogen although there does not appear to be a direct association between this property and the production of hypervolemia (Furth and Sobel, 1946). Preliminary experiments on sustained administration of large doses of stilbesterol and natural estrogens have failed to elicit hypervolemia. The granulosa cell tumor may well be capable of secreting other substances as precursors of the estrogenic material or perhaps an independent substance or a metabolite of substances secreted is responsible for the hypervolemia.

Hormonal regulations of blood volume. It is known that several steroids can cause retention of salt and water (Harrop, 1937; Thorn, Nelson and Thorn, 1938) and that desoxycorticosterone acetate causes an increase in blood volume (Clinton and Thoon, 1943). Various endocrine imbalances have been known to alter somewhat the blood volume. The plasma volume is decreased in myxedema (Gibson and Harris, 1939) and in adrenal insufficiency (Rowntree and Brown, 1929). It is increased in hyperthyroidism (Gibson and Harris, 1939). The increase in blood volume and the retention of water seen in liver disfunction may also be due to an endocrine imbalance (Labby and Hoagland, 1947).

The increase in blood volume found in these conditions is less severe than the hypervolemia of mice bearing granulosa cell tumors and differs quantitatively from it. An increase of the extracellular-

extravascular space is not part of hypervolemic process secondary to granulosa tumors. It is thus probable that the mechanism of the production of hypervolemia in mice bearing the granulosa cell tumor differs from the known physiological changes in body water and blood volume and is dependant on a hitherto unidentified principle.

The relation of hypervolemia to increased circulating albumin. In nearly all instances the plasma albumin concentration in hypervolemic mice is normal and therefore the amount of total circulating albumin is increased. This raises the question as to whether the hypervolemia caused an increase in circulating albumin or whether it is secondary to heightened plasma albumin synthesis. It is interesting to note in this connection that estrogens have been observed to increase plasma albumin (Levin and Leathem, 1942).

Starling's theory requires that a decreased or increased concentration of serum albumin be followed by a decreased or increased blood volume respectively so as to maintain the capillary blood pressure unchanged. A dynamic equilibrium exists between plasma protein and tissue proteins (Holman, Mahoney and Whipple, 1934); and an ever increasing rise in the amount of albumin in the blood might be expected to increase the blood volume.

There appears to be no information on the influence of repeated injections of excessive amounts of albumin on the plasma volume. Heyl, Gibson and Janeway (1943) have shown that a blood volume rise followed the single administration of a concentrated solution of human and bovine plasma albumin to men who were bled 10–20% of their total blood volume. The increase was in agreement with the values expected from in vitro studies of the osmotic pressure of these substances.

Elman and Davey (1943) obtained an increase in plasma protein after a week of transfusion of large quantities of plasma into dogs with dietary hypoalbuminemia. Both plasma albumin and globulin were increased, mostly the former. There was also a striking increase in plasma volume, so that the total circulating albumin doubled in all experiments. Holman (1942) also obtained increased plasma volume after repeated injections of homologous plasma into dogs.

No clinical condition is known to be associated with hyperalbuminemia. This would, however, be obscured by an increase in plasma volume and since blood volume determinations are not made systematically the hyperalbuminemia would be overlooked.

It needs emphasis that the material in the albumin fraction of the hypervolemic mice has not yet been shown to be albumin only and an electrophoretic study of the plasma is desirable.

Possible relationship to V.D.M. A puzzling feature of this hypervolemia is the selected and variable involvement of adrenals, liver and spleen. While the relative distribution of erythrocytes and plasma in various organs of the hypervolemic animal has not yet been deter-

mined, it is obvious from morphological data that by far the greater portion of the increased blood volume is in the liver. According to Maximow and Bloom (1944), the sinusoids of the adrenal cortex resemble those of the liver and it is possible that both respond to the same specific substance.

Grab, Janssen and Rein (1929) have shown that the injection of 1/100–1/20 mg. of adrenaline into the dog produced an immediate expulsion of blood from the liver amounting to 26–59% of the weight of that organ. The analogous behavior of the adrenal does not appear to have been reported. A vasodilator substance or one which inhibits the above effect of adrenaline upon the liver could be responsible for the congestive changes. This suggested a search for a substance similar to the *V.D.M.* of Schorr, Zweifach, and Furchgott (1945). These workers have assayed the tumors, blood and livers of the hypervolemic animals and their findings will be presented elsewhere. It appears from their studies that an excess of *V.D.M.* may come from the liver of the hypervolemic mouse but not from the tumor.

If a vasodilator substance is slowly elaborated by the tumor-bearing animal a secondary increase of blood volume can be anticipated (Bard, 1941). In the light of this hypothesis this hypervolemia is in essence a mild protracted shock.

The recent studies of Clark and associates (1947) have shown that malignant tumors in man are associated with a reduced blood volume. They did not test granulosa tumors. The concept presented above would contradict current ideas assuming that chronic shock is associated with a reduced blood volume.

Possible relation to the function of the hepatic sphincter. Moutner and Pick (1929) have demonstrated the presence of a sphincter in the hepatic vein which is constricted by histamine and released by adrenaline in the dog but not in the goat or cat.[8]

If the congestive changes were caused by sphincteral constriction of the hepatic vein it would be expected that congestion in the liver be of the central type and that there would be a marked passive congestion of the spleen and intestine. Although splenic congestion does occur with hypervolemia, it can be slight and mesenteric congestion is rare. Furthermore, the gonads and bone marrow, which are not in the portal system, are also slightly or moderately congested. The adrenals can be extremely congested in the presence of slight liver congestion.

SUMMARY AND CONCLUSIONS

Repeated blood volume determinations were made on mice bearing transplanted granulosa cell tumors. The blood volume begins to rise after the tumors have reached a moderate size. Once this process is set in motion the blood volume increases rapidly and consistently.

[8] Liver congestion was not observed in 2 mice bearing subcutaneous pellets of 50 mg. of histamine dihydrochloride in 100 mg. of beeswax.

This hypervolemia is not accompanied by an increase in the extracellular-extravascular space.

The blood urea levels may be high or normal in the presence of advanced hypervolemia. The prothrombin values are frequently above normal, both liver and kidney seem to be functioning normally at onset of hypervolemia.

The plasma protein concentration may be normal and when it is reduced this is due to a decrease of plasma globulin concentration. The absolute amount of plasma albumin is increased and this increase roughly parallels the degree of hypervolemia. Further studies are needed to determine if this rise in albumins is the cause or consequence of hypervolemia and to establish the site of this albumin production.

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EFFECT OF ULTRAVIOLET IRRADIATION ON BIOLOGICAL ACTIVITY OF α-ESTRADIOL BENZOATE[1]

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SMITH AND SMITH (1944, 1945, 1946, 1947) have advanced the theory that *in vivo* estrogen degradation products which are themselves relatively inactive on the vagina, may have certain other properties which have been attributed to estrogen *per se*, notably the effects upon the secretory activity of the anterior pituitary.

In this connection they investigated the pituitary stimulating ability of Westerfeld's lactone, an *in vitro* oxidative inactivation product of estrone. Although the lactone was only one-fifteenth as active as estrone with respect to vaginal estrus, it was reported to be more active than estrone in causing the release of luteinizing hormone from the pituitaries of immature female rats. It was also found to be quite active in reducing the transplantable pituitary gonadotrophic activity and in increasing the adrenal and pituitary weight of male rats. This activity was reported to be much greater than could be accounted for on the basis of the slight estrogenic activity of the lactone. Moreover, this activity was exhibited in either intact or castrate male rats, whereas estrone exhibited similar activity only in castrate male rats.

The Smiths interpret this difference as indicating that the lactone exerts its effect by directly stimulating the pituitary, whereas estrone acts indirectly through the formation of the lactone or some similar compound whose production is inhibited by testicular secretion. They point out that testosterone, progesterone and adrenal cortical extract all reduce the rate of formation of a non-estrogenic urinary estrogen degradation product which they find in amounts accounting for 10 to 60 per cent of the estrone administered to women. They found that this urinary component, although not made estrogenically active by simple acid hydrolysis, is, like Westerfeld's lactone, reactivated by zinc-hydrochloric acid hydrolysis.

Simpson and Williams (1946) and Bradbury (1947), on the other hand, have published results which do not appear to be in complete agreement with the work of Smith and Smith.

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[1] Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1103.

Figge (1943, 1945) irradiated an alkaline aqueous solution of estrone with unfiltered light from a quartz mercury arc. When assayed on ovariectomized rats, the irradiated estrone gave only occasional positive vaginal smears at doses up to 6.4 micrograms whereas the non-irradiated control estrone solution produced estrus in 100 per cent of the animals at levels of 1.6 micrograms and above. However, when 50 micrograms of the irradiated estrone was injected into intact female mice, estrus of 10 to 14 days duration ensued, or usually 2 to 3 days longer than in the controls which received a corresponding dose of non-irradiated estrone. When the solutions were again tested, this time on ovariectomized mice, the irradiated estrone was found to be "relatively inactive" as compared to non-irradiated estrone, although dosages were not stated. The apparent failure of the irradiated product to produce estrus in ovariectomized females while producing estrus in intact female mice was interpreted on the basis of the theory of Smith and Smith. That is, irradiation of estrone was assumed to have destroyed its direct vaginal stimulating activity while enhancing its ability to stimulate the pituitary to increased gonadotrophic activity.

The present work represents an attempt to investigate the possibility that ultraviolet irradiation of the estrogens, although decreasing their vaginal stimulating activity in ovariectomized animals, might enhance their effectiveness in the intact animal, by increasing their effectiveness as stimulants of pituitary gonadotrophic activity. In addition, mammary stimulating activity was also observed before and after irradiation.

Allen and Ellis in 1925 observed that ultraviolet irradiation of corn oil solutions of hog follicular extract destroyed their vaginal stimulating activity, but no mention was made of pituitary stimulating activity.

Butenandt *et al.*, (1941, 1942, 1943) have extensively studied the chemistry of the ultraviolet irradiation of several of the steroid sex hormones. In the case of estrone they have identified in the irradiation mixture the 13-epi-isomer of estrone, to which they gave the name lumiestrone. They found this to be inactive in 10 microgram doses in the Allen-Doisy test, but gave no information regarding pituitary stimulating activity. The yield of this isomer was relatively small, and appreciable amounts of unchanged estrone were also recovered from the irradiation mixture.

METHODS

Two ml. of a sesame oil solution of α-estradiol benzoate[2] were placed in the inverted lid of a 10 cm. petri dish. The solution contained 0.33 mg. of the estrogen per ml. This was placed 12 inches from the mercury arc of an ultraviolet lamp (Hanovia, 110 volts, 60 cycles, 5 amps.) and irradiated for a period of 24 hours. During this process the temperature at the petri dish rose to 50°C. and the oil became very viscous but remained clear. Next,

[2] Courtesy of Schering Corporation, Bloomfield, N. J.

1.65 ml. of the oil was recovered and diluted with olive oil to give a concentration of 200 micrograms per ml. (figured on the basis of 330 micrograms per ml. of the irradiated oil recovered). This solution was still clear and somewhat viscous. From it weaker dilutions were made using olive oil. At 20 micrograms per ml. a fairly heavy flocculent precipitate resulted, which was very slow in settling out. At 10 micrograms per ml. the precipitate was lighter, at 2 micrograms per ml., barely detectable, and at 0.2 micrograms per ml., not visible. Such precipitate formation has not been observed to occur upon dilution of non-irradiated sesame oil solutions of this estrogen with olive oil.

The ovariectomized mice used for vaginal assay, with the exception of those indicated by an asterisk in Table 1, were primed with 0.1 micrograms of α-estradiol benzoate. Any animals that did not respond to this level of

TABLE 1. VAGINAL RESPONSE OF OVARIECTOMIZED FEMALE MICE TO α-ESTRADIOL BENZOATE BEFORE AND AFTER ULTRAVIOLET IRRADIATION

Total dose, micrograms	Estradiol benzoate		Irradiated estradiol benzoate			
	No. mice	% positive	No. mice	% positive	No. mice	% positive
10.	19	100*	14	100		
1.	16	100*	14	86	14	64*
0.5	17	94*	14	43	14	43*
0.1	178	81*	24	0		
0.08	16	81				
0.06	16	69				
0.05	16	56				
0.04	15	33				
0.02	14	0				
0.01	15	0				

* These figures obtained on unselected, unprimed animals. All other data obtained on primed animals that had responded with a positive smear to 0.1 microgram of α-estradiol benzoate.

estrogen were withdrawn from the assay groups. Ten to 14 days after priming the mice were used for assay. On day No. 1 a single injection in 0.05 ml. of oil was given. Smears were taken morning and evening on days 4 and 5.

To test the irradiated and non-irradiated estrogen solutions in intact female mice, groups of normal female mice were smeared regularly. After observing the vaginal smears for a number of days, the animals were injected. The incidence of positive vaginal smears per day before and after injection was tabulated.

For mammary growth assay intact male albino mice were used. In order to make the mammary assay as comparable as possible with the vaginal assay, the mice were given a single injection in 0.05 ml. of oil on day No. 1 and killed on days 5 or 7. Two groups were injected on days 1 and 5 and killed on day 9. The formation of growing end-buds on the mammary rudiments was taken as criterion for positive response. In the strain of mice used the males show a low incidence of spontaneous end-bud occurrence (3 out of 18 mice run as controls with these experiments).

RESULTS

As seen in Table 1, ultraviolet irradiation resulted in a loss of all but 10 per cent of the original vaginal stimulating activity present in

TABLE 2. VAGINAL RESPONSE OF INTACT FEMALE MICE TO α-ESTRADIOL BENZOATE BEFORE AND AFTER ULTRAVIOLET IRRADIATION

Day No.	Group I (21 animals)		Group II (17 animals)		Group III (24 animals)	
	Treatment	% of positive vaginal smears	Treatment	% of positive vaginal smears	Treatment	% of positive vaginal smears
1		43		12		12
2						25
3				18		0
4		14		12		42
5		14		24		42
6				18		33
7		7		29		33
8		19		6		4
9		24		12		12
10				24		33
11		24		29		37
12		29	0.1 μg. non-irradiated estrogen	12		37
13		10		18	0.1 μg. non-irradiated estrogen	17
14		14		29		12
15		14		65		50
16				24		71
17			0.1 μg. non-irradiated estrogen	12		62
18		19		12		26
19	0.1 μg. irradiated estrogen	29		35		13
20		19		76	0.1 μg. irradiated estrogen	13
21		10		59		17
22		24		24		9
23		24		12		9
24		14	0.1 μg. irradiated estrogen	12		26
25		5		35		22
26		14		18		
27		19		6		17
28		5		18		13
29		10		6		33
30		14				
31		10		12		
32				29		
33				25		

the oil solution, as judged by the amounts required before and after irradiation to give a positive vaginal smear in approximately 50 per cent of the ovariectomized mice.

To study the possibility of enhanced gonadotrophic stimulating activity, in intact female mice, the 0.1 microgram level was chosen. As seen from Table 1 this was the highest level of the irradiated product that could safely be given without causing direct vaginal stimulation as a result of residual estrogenic activity. If the 90 per

cent loss of direct vaginal stimulating activity was accompanied by conversion of the estrogen to a pituitary gonadotrophic stimulant, this level of irradiated estrogen should cause an increased incidence of positive vaginal smears in intact female mice. However, as seen in Table 2, 0.1 microgram of the irradiated estrogen caused no increase in the daily incidence of positive vaginal smears, whereas the same level of the non-irradiated estrogen caused a pronounced increase within 72 to 96 hours. Irradiation therefore decreased the vaginal stimulating activity of the estrogen in both intact and ovariectomized mice.

Comparison of the mammary stimulating activity of the irradiated and non-irradiated estrogen is presented in Table 3. The unevenly

TABLE 3. MAMMARY RESPONSE OF INTACT MALE MICE TO ULTRAVIOLET-IRRADIATED AND NON-IRRADIATED α-ESTRADIOL BENZOATE*

Total dose, micrograms	4 days				6 days				8 days			
	Estradiol benzoate		Irradiated estradiol benzoate		Estradiol benzoate		Irradiated estradiol benzoate		Estradiol benzoate		Irradiated estradiol benzoate	
	No. mice	% positive	No. mice	% positive	No. mice	% positive	No. mice	% positive	No. mice	% positive	No. mice	% positive
1.	10	90	10	50	10	80	10	60				
0.1	10	80	13	85	9	77	8	25				
0.05	11	36	13	54								
0.02									12	33	13	46
0.01	11	9	11	36	11	36	12	33				

* The formation of growing end-buds was considered a positive response. Male mice of the strain used show a low incidence of spontaneous end-bud occurrence. Two groups of 9 untreated controls showed 22 and 11 per cent positive respectively.

graded nature of the response to the irradiated estrogen makes evaluation of the results difficult. In general, at the higher dosage levels the irradiated estrogen appears less active than the non-irradiated estrogen. At the lower dosage levels the response to the irradiated estrogen appears to be as good as, or better than the response to non-irradiated estrogen. The spontaneous occurrence of a small percentage of "positive" mammary glands among the untreated control animals, however, detracts somewhat from the significance of the results on the lower dosage levels.

DISCUSSION

The present work confirms the reduced vaginal stimulating activity resulting from ultraviolet irradiation of estrogens. However, within the limits of the differences in techniques and materiel, it fails to support Figge's interpretation of enhanced pituitary stimulating activity as indicated by vaginal cornification in the intact animal. Figge's results may perhaps be explained on a dosage basis. The

dosage of 50 micrograms which he observed to cause vaginal cornification in intact mice was considerably greater than the dosages which were observed to be inactive in ovariectomized rats. In view of the work of Butenandt who always found unchanged estrone in his irradiation mixtures, it would appear that Figge's results can readily be explained on the basis of unchanged estrone.

Since the present work employed α-estradiol benzoate instead of estrone, it cannot be compared strictly to the work of either Figge or Butenandt. It is apparent, however, that any work ascribing pituitary stimulating activity to an estrogenically inactive irradiation product of an estrogen must either be done on a purified irradiation product (such as Butenandt's lumiestrone), or else must demonstrate quantitatively that the irradiation mixture possesses a pituitary stimulating capacity greater than can be accounted for on the basis of residual estrogenic activity.

SUMMARY

Ultraviolet irradiation of an oil solution of α-estradiol benzoate resulted in a loss of 90 per cent of its vaginal stimulating activity. It was not possible to demonstrate in the irradiated product an enhanced ability to stimulate pituitary gonadotrophic secretion as measured by vaginal cornification in the intact female mouse.

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HISTOLOGICAL OBSERVATIONS ON THE RELATION OF INSULIN TO THE DEPOSITION OF GLYCOGEN IN ADIPOSE TISSUE[1]

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THE ADIPOSE tissue of animals in normal nutritional balance contains no histologically demonstrable glycogen. However, Gierke 1906 found that glycogen is deposited in the fat cells of guinea-pigs refed after a period of fasting. The occurrence of glycogen in the fat of other animal species under similar experimental conditions has been confirmed by several European investigators (Arndt, 1926, Wertheimer 1928, Richter 1931, Eger 1938, 1942). Among these Arndt, using dogs, also reported a transient accumulation of glycogen in the fat cells after insulin administration. These old observations take on new interest in the light of recent advances in our knowledge of the biochemistry of adipose tissue (Hook and Barron 1941, Mirski 1942), and the relation of insulin to lipogenesis (Drury 1940, Stetten and Klein 1946). We have therefore undertaken to investigate, with an improved staining method, the deposition of glycogen in the brown and white adipose tissues of normal and diabetic rats, rats given insulin, and rats refed after a period of fasting.

MATERIAL AND METHODS

Forty-five male and female rats of the Long-Evans strain weighing 200–250 gms. were used. The deposition of glycogen in adipose tissue was studied in rats given food freely for 48 hours after a five-day period during which food was withheld. To determine the influence of a high intake of carbohydrate on this process, some of the animals were given pure dextrose during the feeding period while others fed on their usual diet of Purina dog-chow which contains approximately 70 per cent carbohydrate absorbable as dextrose. The effect of insulin deficit on the deposition of glycogen was studied on rats made diabetic by intraperitoneal injection of 200 mg. per kilogram of alloxan monohydrate (Eastman) into animals fasted 48 hours. The effect of insulin excess was investigated on animals sacrificed 24 hours after receiving 5 units protamine-zinc-insulin (Lilly) subcutaneously. Interscapular brown-fat and the adjacent subcutaneous white-fat were removed together and fixed in a mixture of absolute alcohol, formalin and picric acid (Ross-

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man's fluid). Sections were stained for glycogen by McManus' periodic acid technique. This method was originally devised as a stain for mucin (McManus 1946), but in applying it to a variety of tissues in this laboratory it was found that glycogen also stains very intensely. Deparaffinized sections are immersed in a 1% solution of periodic acid for five minutes, then transferred to Schiff's reagent for fifteen minutes. After treating for ten minutes in a solution of sulphurous acid, the sections are washed, then cleared and mounted in the usual sequence of alcohols, xylol, and clarite. Glycogen is stained an intense purple, mucin is somewhat less deeply stained, while collagen, elastic tissue, and a variety of other tissue components are stained pink. Either orange G or light-green are suitable counterstains, although ordinarily none is needed, owing to the pink background of non-specific staining produced by the Schiff reagent after periodic acid oxidation. Glycogen is easily distinguished from mucin by digestion of control sections with saliva. After periodic acid treatment, glycogen was often easily discernible in tissues which were negative or only faintly positive by other methods. The Bauer-Feulgen and Best's carmine stains were employed to some extent in the early part of this investigation, but they were soon abandoned in favor of the McManus periodic acid method because of its more uniform results, greater sensitivity, and more intense color. In a recently published comparative study of several methods for the demonstration of glycogen, Lillie (1947) also found that the use of periodic acid followed by the Schiff reagent was the method of choice. A comparison of the Bauer-Feulgen, Best's carmine, and McManus methods on sections from the same block is presented in figures 1, 2, and 3.

OBSERVATIONS ON GLYCOGEN IN ADIPOSE TISSUE

In addition to white or yellow adipose tissue which occurs in all well-nourished mammals, many species possess gland-like masses of so-called brown-fat located most commonly in the perirenal, interscapular, and axillary regions. In the rat, this tissue is readily distinguishable grossly from ordinary fat by its lobular arrangement and its color, which ranges from light tan to brown. There are also marked differences in microscopic appearance. In ordinary fat cells the cytoplasm is reduced to a thin layer enveloping a single large globule of neutral fat, while the nucleus is displaced to the periphery and flattened. The cells of brown fat contain multiple droplets of neutral fat in a rather abundant, coarsely granular cytoplasm. The nucleus is round and centrally situated.

Rats in normal nutritional state: Traces of glycogen were found occasionally in a few scattered cells of the brown fat of normal animals fed on dog-chow but ordinarily both types of adipose tissue were entirely devoid of stainable glycogen. In rats allowed to feed *ad libitum* on pure dextrose the fat was also negative (RC-7, RC-8).

Fasting and refeeding. When rats are permitted to feed after several days of fasting, the amount of glycogen in the fat has been found by chemical analysis to be greatly increased, reaching its maximum about the second day after the onset of feeding. Thereafter the glycogen content gradually falls off, nearing zero by the 4th day

(Tuerkischer and Wertheimer, 1941). These findings were borne out by preliminary histological studies on the fat of animals sacrificed after various periods of refeeding. The majority of the observations reported under this heading was made therefore on rats fasted for five days, then given access to food for forty-eight hours. Under these experimental conditions glycogen was regularly found in conspicuous amounts in both types of fat.

In the white fat, glycogen occurred in the form of coarse, irregular granules or aggregates around the entire circumference of the thin layer of cytoplasm between the cell membrane and the contained fat vacuole (fig. 4).

In white fat the film of protoplasm around the periphery of the cell is so thin that it is not discernible in ordinary histological sections and histologists have been obliged to devise special methods to demonstrate its existence. These methods either involve the use of hypotonic solutions to cause swelling of the layer of protoplasm by imbibition of water (Bremer, 1938), or take advantage of the fact that some fat cells will store trypan-blue in their rim of cytoplasm after repeated injections of the dye over a long period (Dogliotti, 1928). These relatively elaborate and unphysiologic methods are no longer necessary, for when fat from a rat under the stated conditions is stained by the periodic acid technique, the presence of a thin film of cytoplasm around the periphery of the fat droplet is clearly revealed by the presence of glycogen. This observation is of further interest because it serves to reemphasize the fact that a layer of cytoplasm may be thinned out to the point of invisibility with the optical microscope and nevertheless retain the functional capacity for carrying out complex biochemical processes such as the synthesis of glycogen.

Brown fat is known to have a higher rate of oxygen consumption than white fat (Fleischmann 1929, Hook and Barron 1941) and recent studies using radioactive phosphorus have demonstrated that it also has a much more rapid phosphorus turnover (Littrell, Martin and Hartman 1944). A difference in the physiological activity of the two adipose tissues is also apparent in their response to fasting and refeeding. Although qualitatively the responses of the two kinds of fat were usually the same, glycogen was found to appear in distinctly larger amounts in the brown fat where it occurred as an abundant granular deposit in the cytoplasm between fat droplets. The cells of brown fat were not all equally active, for cells heavily laden with glycogen were often found adjacent to cells which contained none or only very small amounts (Fig. 5 and 6). Differences in amount of glycogen from one animal to the next were easier to evaluate in the brown fat because of its more abundant cytoplasm and greater content of glycogen. Animals are compared in table 1, therefore, on the basis of the amount of saliva-digestible, Schiff-positive material found in their interscapular brown fat. Occasional animals (viz. R-5

TABLE 1. THE GLYCOGEN CONTENT OF BROWN ADIPOSE TISSUE

	Animal number	Rat Chow ad libitum	Dextrose ad libitum
A. Control rats	RC-1	0	
	RC-2	+	
	RC-3	0	
	RC-4	0	
	RC-5	+	
	RC-6	0	
	RC-7		0
	RC-8		0
B. Rats fasted 5 days, then fed 48 hours	R-1	+++	
	R-2	+++	
	R-3	+++	
	R-4	++++	
	R-5	++	
	R-6	+++	
	R-7	++++	
	RS-1		++++
	RS-2		+++++
	RS-3		++
	RS-4		+++++
C. Rats on forced feeding by stomach tube.	RH-1		++ (300)*
	RH-2		+++ (368)
	RH-3		++++ (466)
	RH-4		++++ (310)
D. Rats killed 24 hrs. after receiving protamine-insulin:	RI-1	++	
	RI-2	++	
	RI-3	+	
	RI-4	+++	
5 units	RI-5	++	
	RI-6	++	
	RI-7	+++	
	RI-8	++	
	RIS-1		++++
	RIS-2		+
	RIS-2		++++
	RIS-4		++++
	RI-9	+++	
10 units	RI-10	+++++	
	RI-11	++++	
	RI-12	+++++	
E. Rats with alloxan diabetes	RD-1	0 (562)*	
	RD-2	0 (390)	
	RD-3	0 (284)	
	RD-4	0 (262)	
	RD-5	0 (176)	

* Blood-sugar level in milligrams per 100 cc.

and RS-3) showed more glycogen in the white adipose tissue than in the brown but these were quite exceptional.

When rats had access only to dextrose during the refeeding period, greater amounts of glycogen were found in their fat than when they fed on dog-chow (figs. 8 and 9). Hence the quantity of glycogen deposited appears to depend to some extent upon the carbohydrate content of the diet.

Since adipose tissue is specialized for the storage of nutrients, it might be supposed that its cells were able to store carbohydrate as well as fat during a period of alimentary hyperglycemia such as might be expected to result from voracious eating after a prolonged fast. The effect of hyperglycemia was therefore investigated in rats which were force-fed large amounts of pure dextrose and in rats with alloxan diabetes.

Alimentary Hyperglycemia: Hyperglycemia was produced in two normal rats by giving a 50% solution of dextrose in water by stomach-tube. Four feedings of 2 cc. were given in the first 24 hours and 5 feedings of 4 cc. in the second, a total of 14 gms. of dextrose in 2 days. Blood sugars on the second day were 300 and 368 mgs. percent. Sections of the brown fat of these animals contained considerable glycogen (RH-1 and RH-2). The adjacent white fat was entirely negative. Two other rats which received 20 gms. of dextrose by stomach-tube in 2 days (RH-3 and RH-4) had blood sugars of 310 and 466 mgs. per cent when sacrificed and showed large amounts of glycogen in the brown fat and also a moderate amount in the white fat.

Alloxan diabetes: In 2 rats diabetic for 4 to 6 days (RD-1 and RD-2) with blood sugar levels of 390 and 562 milligrams per 100 cc. of blood, the fat was negative for glycogen (fig. 10). Since diabetic rats do not tolerate prolonged fasting, 4 animals (RD-3–RD-6) were fasted 2 days and then injected with alloxan. Food was withheld for 2 more days. One animal succumbed. The three which survived the 4 day period of fasting were then allowed to feed ad libitum for 48 hours (RD-3, 4, 5). Their blood-sugar levels were somewhat lower than those of the other diabetic animals probably because appetite was impaired by the ketosis associated with fasting. The fat of these rats was also devoid of glycogen. Thus neither hyperglycemia nor fasting and refeeding causes glycogen to appear in the fat of diabetic rats. Hence the occurrence of glycogen in adipose tissue, like the deposition of glycogen in liver and muscle, appears to depend upon insulin.

Insulin administration to normal rats. Richter 1931 was unable to demonstrate glycogen by histological methods in the fat-cells of

EXPLANATION OF PLATE 1

FIGS. 1, 2, 3. Parallel sections from the same block of adipose tissue stained by three commonly used methods for the demonstration of glycogen. Fig. 1.—Bauer-Feulgen method, counterstained with light green. Fig. 2.—Best's carmine stain, counterstained with hematoxylin. Fig. 3.—Periodic acid-Schiff reaction, no counterstain. All three photomicrographs were made with a Wratten B filter and given identical exposure. ×160.

FIG. 4. Ordinary white adipose tissue of a rat. The glycogen appears as coarse granules of varying size in the narrow rim of cytoplasm around the periphery of the fat vacuole. ×440.

FIG. 5. Interscapular brown fat of a rat. Coarse granular deposits of glycogen are found in the cytoplasm between fat vacuoles. ×440.

FIG. 6. Interscapular brown fat of a rat given insulin. Many cells are very heavily laden with glycogen while adjacent cells may contain little or none. ×440

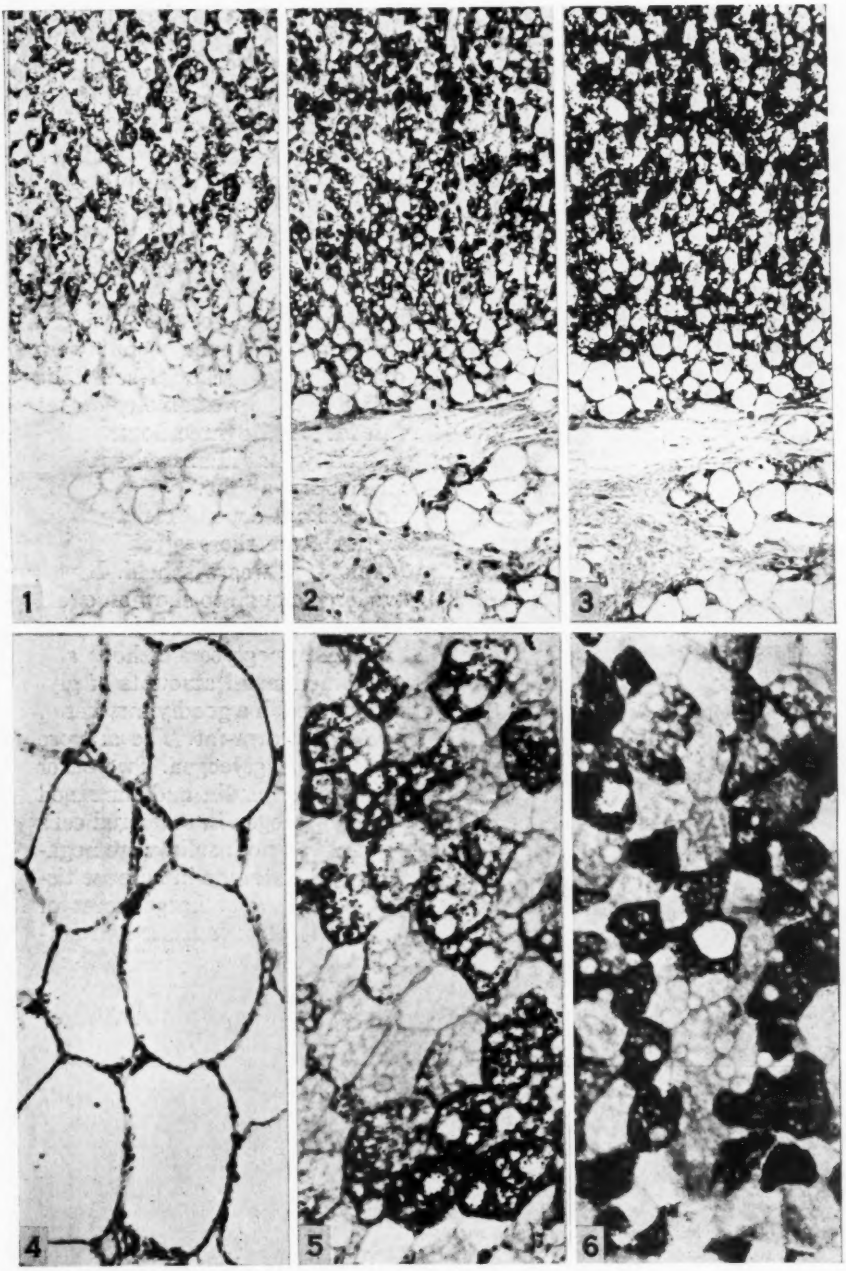


PLATE 1

rats given insulin. Wertheimer 1945, however, using chemical analysis, reported that the glycogen content of the adipose tissue of rats increases after administration of insulin. These chemical observations were fully confirmed by our histochemical studies (Table 1-D). Twenty-four hours after subcutaneous injection of five units of protamine zinc insulin, a considerable amount of glycogen was found in the fat cells (fig. 11). The quantity of glycogen deposited in the brown fat was very striking indeed when the rats had access to pure dextrose during this 24 hour period (fig. 12). Injection of ten units of protamine zinc insulin had a much greater effect than five units. (Table 1-D).

Wertheimer contended that the effects of insulin on the glycogen content of fat were not secondary to increased appetite, but were rather the result of a direct action of insulin upon the fat-cells. His reason for this belief rested upon the fact that he was able to detect a change in the amount of glycogen in fat within three hours of injecting insulin and he thought it was unlikely that increased appetite could have a measurable effect on fat in so short a time.

To verify that the action of insulin does not depend merely upon increased appetite or increased absorption from the gastrointestinal tract, insulin was given to fasted rats and food was withheld. It was thought that the animals might survive long enough to show an effect of the insulin. A rat fasted 8 hours was given 10 units of protamine insulin. Four hours later the animal was in hypoglycemic shock and was sacrificed. Its interscapular fat contained small amounts of glycogen in the cells of the white adipose tissue and in a goodly number of the cells at the periphery of the lobules of brown-fat. The adipose tissues of the control rat were entirely devoid of glycogen. Two other rats fasted 24 hours received smaller doses of insulin and remained alive for five and nine hours. Both showed glycogen in scattered cells of the brown fat. Their controls which received no insulin were negative. Hence the effect of insulin upon glycogen storage in adipose tissue is not attributable to increased appetite alone. Large doses of insulin apparently increase the glycogen content of muscles and adipose tissues at the expense of liver glycogen.

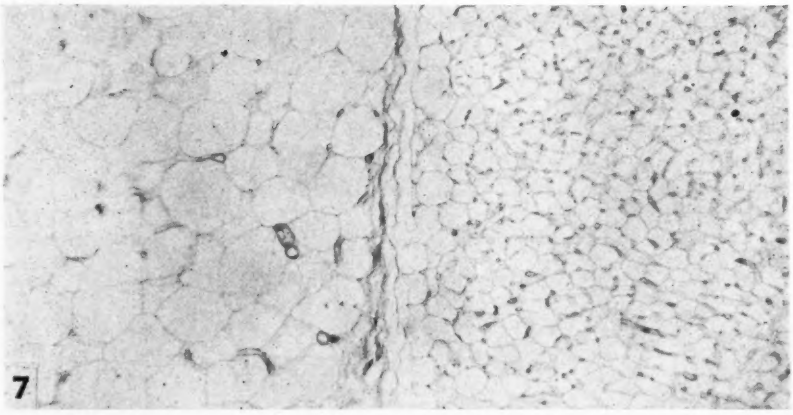
If insulin acts directly upon fat-cells, then a greater effect of a given dose might be expected if the injection were made in the immediate vicinity of the interscapular fat body than if the same dose were

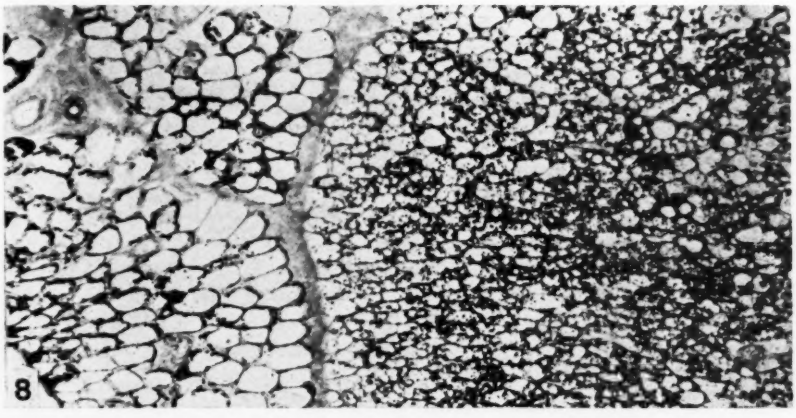
EXPLANATION OF PLATE II

FIG. 7. Interscapular brown fat (right) and adjacent white fat (left) of a normal rat fed on dextrose alone (RC-7). Both types of adipose tissue are negative for glycogen. Stained by the periodic acid-Schiff method for glycogen. ×160.

FIG. 8. Brown fat (right) and ordinary adipose tissue (left) from an animal (R-2) fasted for five days and then allowed to feed for forty-eight hours on Purina dog chow. Glycogen 3 plus. Periodic acid-Schiff method. ×160.

FIG. 9. Brown fat (below) and white fat (above) from a rat (RS-2) fasted for five days and then allowed to feed for forty-eight hours on pure dextrose. Glycogen 5 plus. Periodic acid-Schiff method. ×160.





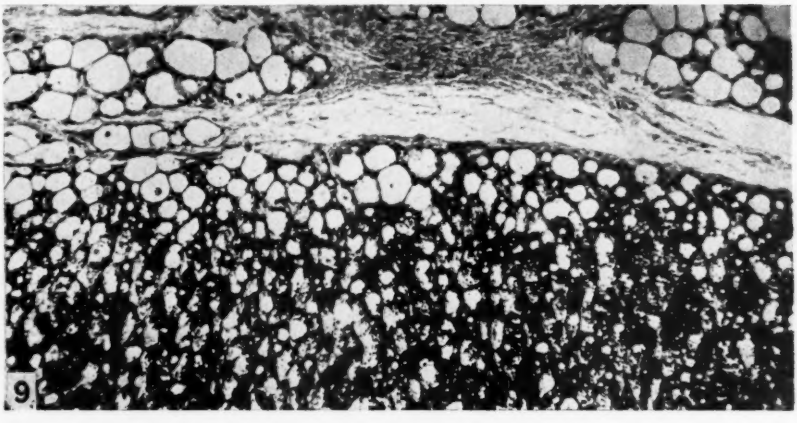
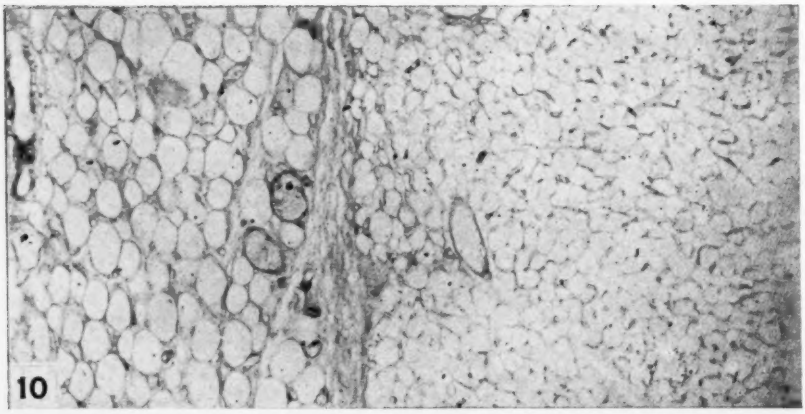
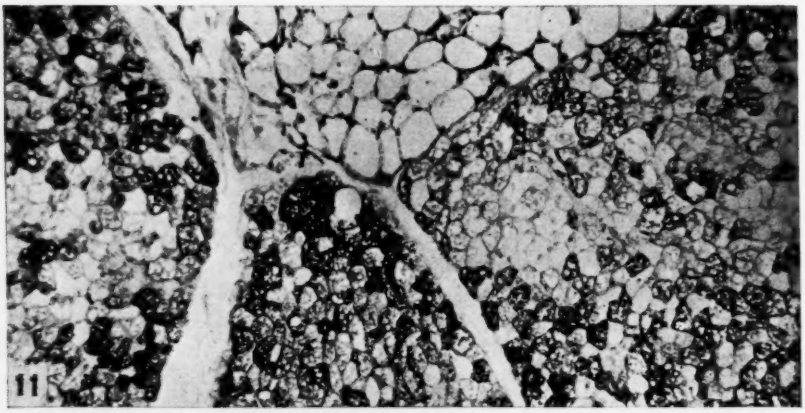


PLATE 2





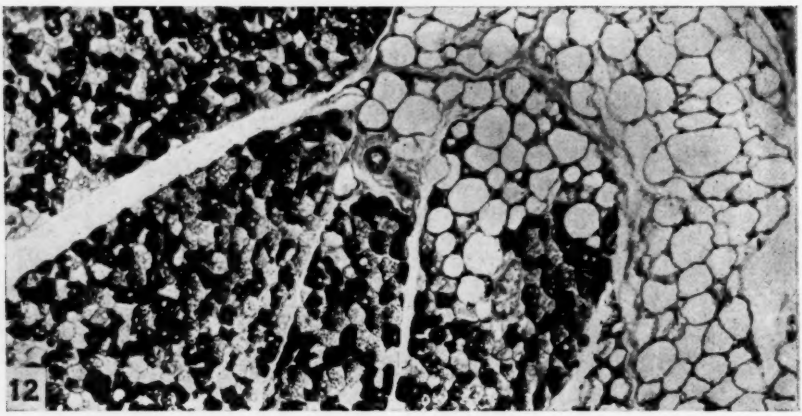


PLATE 3

injected at a distance. Ten rats were divided into pairs of the same weight. One of each pair received a subcutaneous injection of insulin directly over the interscapular fat, the other received the same dose under the skin of the abdomen. Three of the ten pairs of rats received 5 units of protamine-zinc-insulin. With this dose the differences between those injected near the interscapular fat and those injected on the abdomen were inconclusive. In one pair there was definitely more in the brown fat which was at the site of the injection. A second pair showed no significant difference. In a third pair the animal injected on the abdomen appeared to have more glycogen in its interscapular fat than the one injected interscapularly. The latter however showed a very irregular distribution of glycogen, some areas at the periphery of the fat-body (perhaps nearest the site of injection) contained a large amount of glycogen while the remainder showed relatively little. Two other pairs of rats were injected in the same manner with 10 units of protamine-zinc-insulin. With this larger dosage the 2 animals injected in the interscapular region had distinctly more glycogen in the interscapular fat than did the animals injected on the abdomen. The results of these few experiments were not sufficiently consistent to warrant a stronger statement than that they suggest that insulin does have a specific local effect upon fat cells.

The foregoing observation is of interest in view of the clinical observation that diabetic patients occasionally show extensive changes in the subcutaneous fat at the site of insulin injection—a condition commonly called insulin lipodystrophy. In the region involved, areas of fat atrophy and areas of hypertrophy may intermingle. In juvenile patients lipohypertrophy may predominate. Attempts to produce insulin lipodystrophy in normal experimental animals have failed and a specific local effect of insulin in the etiology of this condition has therefore been denied (Reed, Anderson & Mendell 1920, Goldner 1943). The dosage used for daily injection in those studies were based on human dosage per unit of body weight and may have been far too small (0.6 units in rats and 0.01 units for mice). Rats and mice with access to food will tolerate at least 100 times these doses. Our findings on rats indicate that large doses of insulin probably do have a specific local effect on fat cells which may have a bearing upon the problem of insulin lipohypertrophy.

EXPLANATION OF PLATE 3

FIG. 10. Interscapular brown fat (right) and adjacent subcutaneous white fat (left) of a diabetic rat (RD-1) with a blood sugar 562 mg/percent. Both types of adipose tissue are negative for glycogen. Stained for glycogen by the periodic acid-Schiff method. ×160.

FIG. 11. Interscapular brown fat and adjacent white fat of a rat (RI-2) fed dog chow ad libitum for twenty-four hours after receiving a single subcutaneous injection of 5 units protamine-zinc-insulin. Glycogen 2 plus. Periodic acid-Schiff method. ×160.

FIG. 12. Brown fat and white fat of a rat(RIS-1) fed dextrose ad libitum for twenty four hours after receiving a single dose of 5 units of protamine-zinc-insulin. Glycogen 4 plus. Periodic acid-Schiff method. ×160.

DISCUSSION

Numerous physiological investigations have established that in normal animals large doses of insulin tend to inhibit accumulation of glycogen in the liver but cause a decided increase in the glycogen of muscles (Barbour, Chaikoff, McLeod and Orr 1927, Bridge 1938, Swensson 1945). In experiments to determine the effects of exogenous insulin, it has been customary to analyze liver and muscle on the assumption that these were the only "active tissues" involved. It is now apparent from the present histochemical observations, that insulin causes an increase in the glycogen content of adipose tissue as well as muscle. The role of glycogen in muscle physiology is well known, but the significance of glycogen deposited in fat has yet to be elucidated.

Von Gierke 1906 interpreted the glycogen he found in adipose tissue as an intermediate product in the transformation of dietary carbohydrate to depot fat. He thought the occurrence of glycogen in fat-cells during refeeding constituted morphological evidence that this conversion could take place in the fat-cells themselves. These observations attracted little attention because they were contrary to accepted biochemical theory and they were reported at a time when the fat depots were believed to be metabolically inert. Some twenty years later, however, with the discovery that isolated adipose tissue consumes oxygen (Hoffman and Wertheimer 1927), investigations of adipose tissue for evidences of metabolic activity were undertaken with renewed interest. The occurrence of glycogen in the fat of animals fed a high carbohydrate diet after preliminary fasting was confirmed by histological observations (Arndt 1927, Richter 1931) and by chemical analysis (Wertheimer and Hoffman 1927, Scoz 1929, Hausberger and Neuenschwander-Lemmer 1939). In vitro measurements of the respiratory metabolism of fat from animals under these same conditions were found to yield values for respiratory quotient (RQ—Vol. CO_2 output/vol. O_2 intake) which often exceeded unity (Ruska and Quast 1935, Henle and Szpingier 1936, Felix and Eger 1938, Mirski 1942). Since the highest RQ found from combustion of any foodstuff is 1.0 (carbohydrate), higher values are thought to mean that oxygen is being derived from some source other than the respired air. The transformation of carbohydrate to fatty acid would liberate oxygen. It is therefore assumed that values for RQ greater than 1.0 signify the conversion of carbohydrate to fat. Based on this physiological evidence and the earlier morphological and chemical observations of glycogen in fat, the belief became rather widely held in Europe that adipose tissue is capable of synthesizing fat from carbohydrate and that the deposition of glycogen in fat-cells is in some way related to this process.

Investigators in this country, for the most part unfamiliar with the European literature, generally adhered to a more conservative view of the functional capacities of adipose tissue. The new insight

into the intermediary metabolism of dietary fat which has recently been gained through the use of isotopes, has nevertheless forced us to abandon the traditional concept of depot fats as static reserves. The brilliant work of Schoenheimer 1942 and his coworkers has revealed that stored fats are constantly involved in a variety of complex reactions of synthesis, degradation and interconversion. It is still the consensus of opinion, however, that these elaborate molecular reorganizations of depot fat as well as carbohydrate-fat synthesis, take place in the liver and that the products are continuously transported between liver and fat depots by the blood stream. Although adipose tissues are not generally believed to participate in metabolic processes to any important extent, the possibility that they have some part to play in the synthesis and degradation of fatty-acids has not been excluded. The recent studies of Tepperman, Brobeck and Long (1943) indicate that under certain circumstances extrahepatic mechanisms for the conversion of carbohydrate to fat may assume considerable importance. Studying the respiratory exchange of rats with hypothalamic lesions, these investigators found that such animals showed a much greater elevation of R. Q. during glucose absorption than did controls. This suggested that the obesity developed by rats with experimental lesions in the hypothalamus may be the result of a biochemical adaptation by which these animals are enabled to store a greater-than-normal proportion of their food as depot-fat. This change in food utilization is probably brought about by the altered feeding habits of animals with hypothalamic lesions, for Tepperman and his co-workers found a similar elevation of R.Q. in intact animals trained to take their entire ration in only 3 hours of each day. Eviscerate preparations of rats trained in these feeding habits, also exhibited values for R.Q. which were significantly higher than those of the controls. Moreover, when these liverless animals were given added insulin the average R.Q. was greater than one. This was interpreted by Tepperman, Brobeck and Long as evidence for the synthesis of fat by an extrahepatic mechanism which is facilitated by insulin. The tissue in which the synthesis occurred was not identified.

In this connection it is interesting to note that the alteration of feeding habits which Tepperman and his coworkers induced in rats by training consisted essentially of alternating short periods of fasting and rapid feeding and is therefore similar in principle to one of the conditions we have employed to cause glycogen deposition in fat-cells. It has already been shown that isolated adipose tissue from animals fed after previous fasting has an R.Q. greater than one (Wertheimer, 1927; Mirski, 1942). It is suggested therefore that the extrahepatic synthesis of fats which was postulated by Tepperman *et al.*, may take place in the adipose tissue.

When insulin is administered to a normal rabbit, a large increase in hepatic lipogenesis is observed (Stetten and Klein, 1944). The work of Tepperman and others discussed above, suggests that extra-

hepatic lipogenesis may also be increased by insulin injection. In the present investigation insulin administration has been shown to cause glycogen to appear in the adipose tissues of normal rats, particularly in the so-called brown fat. In diabetic rats, on the other hand, where the rate of fatty acid synthesis is known to be only about 5% of normal (Stetten and Boxer), glycogen was never found in the fat cells. Hence both lipogenesis and the occurrence of glycogen in adipose tissue are promoted by insulin excess and prevented by insulin deficit. Inasmuch as the deposition of glycogen in adipose tissue only occurs under experimental conditions, which are known to favor the rapid conversion of dietary carbohydrate to depot fat, it is reasonable to suppose that the glycogen is in some way involved in lipogenesis. The role glycogen might play in the synthesis of fat is somewhat obscure, but it is possible that the local oxidative breakdown of glycogen may provide both a source of energy and structural units for the synthesis of fatty acids in the adipose tissue itself. The transformation of carbohydrate to fat in the depots may not take place to any great extent in normal animals in nutritional balance. It does seem likely, however, that this extrahepatic mechanism for the formation of new fat may assume considerable importance during the period of recovery from a prolonged fast, after ingestion of excessive amounts of carbohydrate, and also in animals receiving a large dose of insulin.

If we are correct in assuming that the glycogen in adipose tissue is related to the conversion of carbohydrate to fat, then it would appear that more fat is synthesized from carbohydrate in brown adipose tissue than in the white. This might be expected to result in a different chemical composition of the fats in the two tissues inasmuch as fats synthesized from carbohydrate are more highly saturated than those derived from other sources (Anderson and Mendel 1928). No values are available for the iodine number of the fats in the brown adipose tissue of the rat but there is some indirect evidence suggesting that brown fat is more highly saturated than ordinary adipose tissue. Rats raised on a vitamin E deficient diet containing 20% cod-liver oil show a progressive accumulation of acid-fast pigment in their white fat depots but the brown fat shows no significant alteration (Mason, Dam, and Granados, 1946). This pigment is thought to consist of polymerized peroxides of long-chain unsaturated fatty acids. The fact that the brown adipose tissue is spared in this process is consistent with the thought that its fats are made chiefly from carbohydrate and are therefore relatively highly saturated. Application of osmic acid staining to both types of adipose tissue provides additional evidence of such a difference in chemical composition. Oleic acid and its compounds are blackened by the reduction of osmium tetroxide to osmium dioxide. Compounds of palmitic and stearic acids, being saturated, are stained only when exposure to osmic acid is followed by treatment with 70% alcohol which changes it to osmium hydroxide—a process called secondary staining. Thus when the secondary staining is omitted the amount of blackening of a fat with osmic acid is a

rough indication of its degree of unsaturation. Brown fat is not blackened by this treatment while the adjacent white fat is. It is suggested, therefore, that the neutral fats in brown adipose tissue are more highly saturated.

SUMMARY

The deposition of glycogen in adipose tissue has been investigated by histochemical methods in normal and diabetic rats, rats given insulin and rats refed after a period of fasting.

The fat cells of normal rats are ordinarily devoid of stainable glycogen. After the administration of a single large dose of protamine-zinc-insulin to normal rats and also during the refeeding of fasted animals, glycogen was regularly present in conspicuous amounts. Under both of these experimental conditions glycogen was much more abundant in the cells of the interscapular and perirenal brown fat that it was in the subcutaneous white fat. Glycogen was invariably absent from the adipose tissues of diabetic rats.

The significance of glycogen in adipose tissue has been discussed in relation to the extrahepatic synthesis of fats from carbohydrate.

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PLASMA INORGANIC IODIDE, A CHEMICAL REGULATOR OF NORMAL THYROID FUNCTION[1]

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IN AN EARLIER communication (Wolff and Chaikoff, 1948) it was shown that the administration of large amounts of iodine temporarily inhibits the capacity of the *normal* thyroid gland to bind iodine organically. This inhibition appeared to be related to the level of plasma iodine. So long as the concentration of plasma iodine exceeded 20–35γ per cent, no organic binding of iodine occurred in the gland, and only when the concentration fell below this critical range did the gland resume its function of depositing iodine in an organic form. On the basis of these findings it was postulated that the level of plasma iodine is part of a homeostatic mechanism governing hormone synthesis in the normal gland. Thus, when a large amount of iodine is ingested, the prevention of its deposition as organically bound compounds in the thyroid gland keeps the ingested iodine circulating as inorganic iodide, in which form it is readily excreted by the kidney. In this way, it was argued, the formation of toxic amounts of the thyroid hormone is prevented.

In the experiments described above, the inhibition of thyroid function was temporary, lasting some 8–17 hours, i.e. during the time when the concentration of plasma iodine remained well above 35γ per cent. The experiments described here provide additional proof that plasma iodine, probably by influencing the level of inorganic iodide in the gland, is an inhibitor of normal thyroid function. It is shown in the present study that the period of inhibition of thyroid function imposed by high iodine administration can be prolonged by preventing the escape of the administered iodine by excision of the kidneys.

EXPERIMENTAL

Long-Evans rats ranging from 171 to 240 gms, were used throughout. The animals were fed until the time of the operation but afterwards were permitted access to water only. The rats were first injected intraperitoneally with 6 mg. of sodium pentobarbital and were then completely anesthetized with ether. The kidneys were exposed by a lateral incision and the perirenal fat peeled off, care being taken

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to avoid damage to the adrenals. The ureter and renal vessels were then tied close to the hilum and the kidneys removed. After the operation the animals were kept in a warm room (28–30°C.). Approximately 1–2 hours after they had recovered from the anesthesia, they were injected with I^{127} labeled with I^{131} and sacrificed at various intervals thereafter. Blood was removed by heart puncture, and total plasma iodine was determined for each rat on 2 cc. of plasma, as described by Taurog and Chaikoff (1946). The gland iodine was separated into organic and inorganic fractions as previously described (Wolff and Chaikoff, 1948).

TABLE 1. THE UPTAKE OF IODINE BY THYROIDS OF NEPHRECTOMIZED RATS FOLLOWING INJECTIONS OF 10 AND 500γ OF I^{127} AS KI AND THEIR CONVERSION TO DIIODOTRYOSINE AND THYROXINE

Time after I^{127} injection	10γ I^{127} Injected				500γ I^{127} Injected			
	Total amount of injected I^{127} found in thyroid	Per cent of injected I^{127} found in thyroid as		Plasma total iodine*	Total amount of injected I^{127} found in thyroid	Per cent of injected I^{127} found in thyroid as		Plasma total iodine*
		Inorganic	Organic			Inorganic	Organic	
hours	γ			γ per cent	γ			γ per cent
4	0.87	11	89	12.3	4.6	99	1.0	178
4	1.5	5	95	13.2	7.5	99	0.39	200
4	1.4	7	93	12.8	5.5	99	0.93	215
10	2.4	3	97	8.9	12.5	99	0.36	225
10	0.91	16	84	8.9	7.0	99	0.84	230
10	0.70	11	89	9.5	11.5	99	0.64	150
18	2.9	4	96	10.5	6.3	99	1.3	161
18	2.0	5	95	12.4	8.9	99	0.79	313
18	1.9	6	94	15.0	2.6	96	3.8	195
26	1.4	3	97	8.8	7.9	99	1.4	185
26	1.9	6	94	9.2	7.6	99	0.92	306
26	2.4	3	97	9.2	5.8	98	2.1	146
32	5.5	2	98	8.9	9.2	99	0.67	105
32	4.1	2	98	9.5	6.8	97	2.9	173
32	4.3	2	98	8.9	8.0	95	4.9	225
40	4.7	2	98	8.5	1.8	74	26	218
40	3.8	2	98	8.0	9.9	96	3.7	180
40	4.4	2	98	8.8	3.9	81	14	200

* At time thyroids excised.

RESULTS

The rats were injected with either 10 or 500γ of I^{127} as KI one to two hours after removal of both kidneys and groups of them sacrificed at intervals of 4, 10, 18, 26, 32, and 40 hours after the injection. Values for total plasma iodine and for the amounts of injected I^{127} recovered in the gland are recorded in table 1. The amounts of injected I^{127} present in the inorganic (trichloroacetic-acid soluble) or the organic (trichloroacetic-acid insoluble) fractions of the gland were obtained by multiplying the numerical proportion of the injected I^{131} recovered in the respective fraction by the gamma of I^{127} injected.

Rats Injected with 10γ of I^{127}.—The highest concentration of I^{127} found in the plasma of rats that had been injected with 10γ of I^{127} was 15γ per cent. Since the kidney is the main pathway of excretion

for inorganic iodide (Keating, Power, Berkson, and Haines, 1947; Nelson, Palmer, Park, Weymouth, and Bean, 1947; Spector, Mitchell, and Hamilton, 1945; Perlman, Chaikoff, and Morton, 1941), the finding of plasma-iodine values of 8–9γ per cent in nephrectomized rats as late as 40 hours after the injection is not surprising.

In this experiment the amounts of the *injected* I^{127} recovered in the thyroid gland increased with time. At the later intervals about 4γ or 40 per cent of the injected dose were found in the gland. *At all intervals studied, approximately 90 per cent of the newly accumulated iodine was organically bound.*

TABLE 2. RELATION OF THE LEVEL OF PLASMA IODIDE TO ORGANIC BINDING OF IODINE BY THE THYROID GLAND
(All values are the averages of the results recorded in table 1)

Interval after injection of I^{127} as KI hours	Plasma iodine γ per cent	Injected I^{127} recovered in gland		
		Total	Organically bound	
		γ	Amount γ	Per cent of total
4	13	1.3	1.2	92
	198	5.8	0.04	0.69
10	9	1.3	1.2	92
	202	10.3	0.06	0.58
18	13	2.3	2.2	96
	223	5.9	0.08	1.4
26	9	1.9	1.8	95
	212	7.1	0.10	1.4
32	9	4.6	4.5	98
	168	8.0	0.22	2.7
40	8	4.3	4.2	98
	199	5.2	0.46	8.9

Rats Injected with 500γ of I^{127}.—The levels of plasma iodine in these rats were always in excess of 100γ per cent and in some were over 300γ per cent. The concentration of plasma iodine remained fairly constant during the entire period of observation (average, 210γ per cent).

Larger amounts of I^{127} were recovered from the glands of the rats that received 500γ than from those of rats injected with 10γ. But in this experiment practically none of the newly accumulated I^{127} was organically bound. For example, in the rats that were sacrificed 26 hours after the injection of 500γ dose, from 5.6 to 7.9γ of the injected I^{127} was found in the gland; yet only 0.07–0.12γ or at most 2 per cent of this iodine was organically bound.

DISCUSSION

In the present investigation 2 plasma levels of iodine, one in the neighborhood of 10γ per cent, the other of 200γ per cent, were estab-

lished and maintained for a period of 40 hours by a single injection of KI into nephrectomized rats. As shown in table 2, which summarizes the results of these experiments, so long as plasma iodine remained in the neighborhood of 200γ per cent, the gland failed to convert iodine to organic forms; even after 40 hours, only 9 per cent of the injected I^{127} found in the gland had been converted to organic forms. That the thyroid gland of the nephrectomized rat retained its capacity to bind iodine organically during the entire period of observation is brought out in the experiments in which the levels of plasma iodine were kept below 15γ per cent. More than 90 per cent of the iodine found in the thyroids of these rats was organically bound. The present investigation thus shows that the period of inhibition of organic binding by the thyroid gland can be extended at will by maintenance of a high plasma-iodine level.

It was pointed out earlier that the inhibition of organic binding of iodine by the gland offers a rational explanation for the response of the thyrotoxic patient to iodine treatment (Wolff and Chaikoff, 1948). This view is fully supported by the results of the present investigation. Since the findings in the normal and nephrectomized rats show that the gland is inhibited only so long as the plasma concentration of iodine is kept high, it is suggested that the failure to maintain high enough levels of plasma iodine may account for the exacerbation of the hyperthyroid state sometimes noted during the treatment of patients suffering from hyperthyroidism.

SUMMARY

The relation of the level of plasma inorganic iodide to the organic binding of iodine in the thryoid gland was investigated. A single injection of 500γ of iodide into bilaterally nephrectomized rats made possible the maintenance of a plasma iodine level of approximately 200γ per cent for a period of 40 hours. Although the injected iodine readily entered the gland, practically none of the newly accumulated iodine was converted to organic forms even after 40 hours. On the other hand, when 10γ of iodine were injected into nephrectomized rats, the level of plasma iodine remained low enough (below 15γ per cent) to permit incorporation of nearly all the newly accumulated iodine into organic forms. The significance of these findings for iodine treatment of thyrotoxic patients is pointed out.

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THE EFFECT OF PARTIAL HEPATECTOMY ON THE *IN VIVO* ACTIVATION OF TRIPHENYLCHLOROETHYLENE[1]

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In 1941 Emmens published the first of a series of extensive studies on the biologic activity of synthetic and natural estrogenic materials. In his early experiments he compared the ability of a wide series of compounds to produce vaginal cornification in spayed mice when given either by subcutaneous injection or by direct application to the vaginal mucosa. These studies yielded results of singular interest, in that the materials studied could rather easily be divided into two classifications: (1) those compounds which required only a small fraction of the subcutaneous dose when the material was given intravaginally; and (2) those compounds which required essentially the same amount of material by either route of administration. All the natural estrogens and their esters which were studied fell into the first group, which Emmens then termed the "estrogens." Diethylstilbestrol also fell into this first group. Triphenylethylene, on the other hand, fell into the second group, which Emmens termed the "proestrogens." The name of this second group was chosen because it appeared to Emmens that the organism had to convert these materials into estrogenically active forms before local activity could be achieved.

Further proof that the organism converted "proestrogens" to "estrogens" with local action was obtained (Emmens, 1942b) when the problem was studied in spayed mice with two separate vaginal sacs. These were prepared by the method of Robson and Adler (1940), who demonstrated that if natural estrogens are instilled locally into one of the vaginal sacs, it alone is cornified. In contrast to this, Emmens was able to show that if "proestrogens" are instilled locally in sufficient amount to cornify one sac, then the other is also cornified.

Stroud (1940) showed that if diphenyl-hexadiene (a "proestrogen") is given to rabbits, a small amount of a phenolic material, probably 4:4′-dihydroxy-ν:δ-diphenyl-β:δ-hexadiene (an "estro-

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[1] Supplied by Merck & Co., Inc. through the courtesy of the late Dr. D. F. Robertson.

gen"), is excreted in the urine. Accordingly, Emmens (1943), studying the S/L (Systemic to Local) ratio of urinary extracts obtained from guinea pigs given various "estrogens" and "proestrogens," observed that if estrogens are administered, the urine has a high S/L ratio but not as high as the administered compound. If certain "proestrogens" are given, the urine possesses an S/L ratio which is much greater than that of the administered material. However, some "proestrogens" failed to increase urinary estrogen excretion. This seems to indicate that the urinary excretion products after "proestrogen" administration consist largely or wholly of "estrogens".

Applying our method of intrasplenic injections to the study of the problem (Segaloff, 1944), we observed that if triphenylethylene was injected intrasplenically with the spleen *in situ*, less material was required for vaginal estrus than if the material was injected subcutaneously or into a spleen divorced from the portal circulation. These observations led us to believe that the "proestrogens" are converted in the liver to estrogenically active materials. Emmens (1942a) noted that triphenylchloroethylene possesses estrogenic activity. However, since by our techniques the activation of triphenylchloroethylene was greater than triphenylethylene and its estrogenic activity was also so much greater, it was selected for further study.

All the "estrogens" except triphenylchloroethylene, which we had studied by our technique (Segaloff, 1943, 1944), showed pronounced inactivation in the liver. In addition to this, we (Segaloff, 1946) noted that partial hepatectomy greatly decreased the hepatic inactivation of α-estradiol. Accordingly, studies were undertaken to determine whether partial hepatectomy would reduce the degree of activation of triphenylcholoroethylene.

MATERIALS AND METHODS

Young adult female rats obtained from Maguran Farms were spayed 3 or 4 weeks prior to the study. Fourteen days after priming with 50 micrograms of estrone in 0.1 cc. of peanut oil injected subcutaneously partial hepatectomy was performed, under ether anesthesia, as in our previous studies. While the animals were still anesthetized, the test injection of triphenylchloroethylene in 0.05 cc. of 5% benzyl alcohol in sesame oil was made either intrasplenically or subcutaneously. Vaginal smears were taken with a moistened cotton-tipped toothpick, 48, 54, 60 and 72 hours after the injection. The smears were air dried and stained with hematoxylin and eosin. A smear which showed only cornified epithelial cells at one or more of these times was classified as representing a positive vaginal estrus response for that animal. Twenty or more animals were employed at each level of hormone administration.

RESULTS

The results are presented in Table 1. The results in the intact animals are in essential agreement with those previously published. Following partial hepatectomy there was a slight decrease in the

effectiveness of the subcutaneously administered triphenylchloroethylene. A great contrast was noted when the results of intrasplenic injection were compared in the two groups of animals. There was a more than three-fold increase in the effectiveness of intrasplenically administered triphenylchloroethylene after partial hepatectomy.

DISCUSSION

The results presented in the preceding paragraph were entirely unexpected; indeed partial hepatectomy affected the activation of triphenylchloroethylene in the opposite direction to that which was predicted on the basis of our experience with α-estradiol (Segaloff, 1946). Using this identical technique with α-estradiol, we observed

TABLE 1. RESULTS OF ADMINISTRATION OF TRIPHENYLCHLOROETHYLENE BY VARIOUS ROUTES TO SPAYED AND PARTIALLY HEPATECTOMIZED SPAYED RATS

	Subcutaneous injection			Intrasplenic injection			Ratio SC:IS
	Number of animals	Dose ug.	Estrus %	Number of animals	Dose ug.	Estrus %	
Controls	20	30	25	38	6	37	
	56	40	52	40	7	50	1:0.17
	25	80	80	20	8	90	
Partial hepatectomy	20	40	20	25	1.5	24	
	24	60	50	33	2	49	1:0.03
	20	80	85	24	6	88	

that it was greatly decreased in estrogenic activity by passage through the liver. Further, it was possible to reduce the degree of hepatic α-estradiol inactivation strikingly by means of partial hepatectomy. Accordingly, since triphenylchloroethylene is activated by passage through the liver, it was our belief that partial hepatectomy would reduce the activation, but as shown in Table 1, this did not occur.

Naturally, there are many possible explanations of this complex phenomenon, but the following seems to apply best to our present knowledge. First, the activation of triphenylchloroethylene is but one step in its metabolism. It is possible that this step is accomplished by conversion of the phenyl radical to a phenolic one by the addition of hydroxyl groups. Such conversions are known to occur, i.e., the liver is able to convert phenyl-alanine to tyrosine (Bernheim and Bernheim, 1944). The organism as a whole is also capable of producing phenolic estrogens from a phenyl "proestrogen" (Stroud, 1940; Emmens 1943). The second step is then the conjugation and/or oxidation of the estrogen formed in the first step. Therefore, under this concept, there would be going on two essentially competing processes which would influence the final estrogenic activity: one to increase it, the other to decrease it. In order to explain the observed phenomena, still another assumption is necessary: namely, that the destructive

process (be it oxidation or conjugation) requires more hepatic tissue and is the limiting factor in the final resultant estrogenic activity. Thus partial hepatectomy decreases still further the amount of liver available for the inactivating phase, thereby permitting the activation to predominate and producing the apparently anomalous phenomenon of increasing the activation of triphenylchloroethylene by partial hepatectomy.

SUMMARY

Triphenylchloroethylene was injected subcutaneously and intrasplenically into spayed female rats. The estrogenic response, as judged by vaginal estrus, was increased when the material had to pass through the liver before entering the systemic circulation (intrasplenic injection).

This hepatic "activation" or potentiation of estrogenic activity was increased by partial hepatectomy.

A tentative explanation of these phenomena is presented.

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NOTES AND COMMENTS

METABOLISM OF THE STEROID HORMONES: ANDROSTERONE ADMINISTRATION TO GUINEA PIGS[1]

In a previous communication it was shown that eight per cent of orally administered androsterone and two per cent of subcutaneously administered androsterone were isolated as isoandrosterone from the urine of normal male guinea pigs (Dorfman, Schiller, and Fish, 1944). The urinary extract from the subcutaneously administered androsterone has been studied further and will be the subject of this report.

EXPERIMENTAL

As reported previously, 80 mg. of androsterone propionate,[2] in 2 cc. of sesame oil, was injected subcutaneously for three successive days into each of six adult male guinea pigs. A total of 1.44 grams of androsterone propionate or 1.2 grams of androsterone was administered. The details of urine collection, extraction, and preparation of the various fractions have been described previously (Dorfman, Schiller, and Fish, 1944). The non-ketonic digitonin precipitable fraction (4.2 mg.) was almost pure cholesterol. The non-ketonic digitonin soluble fraction (89.7 mg.) yielded no pure substances when chromatographed as the acetates using aluminum oxide (Merck).

The ketonic digitonin fraction (109.4 mg.) was subjected to chromatographic analysis as the acetates using 3.3 grams of aluminum oxide (Merck). The column was eluted with carbon tetrachloride, and carbon tetrachloride containing from 0.1 to 1.0 per cent of absolute ethanol. From two elutes containing 0.1 per cent ethanol in carbon tetrachloride there were obtained 3.7 mg. of androsterone (m.p. 179–182°C.) and 5.6 mg. of androsterone (m.p. 178–180°C.), respectively. No depressions in melting points when these samples were mixed with an authentic sample of androsterone. An acetate m.p. 158–160°C. was obtained which when mixed with androsterone acetate (m.p. 160–161°C.) melted at 159–160°C.

SUMMARY

The subcutaneous administration of androsterone to normal male guinea pigs gave rise to isoandrosterone (2 per cent) and androsterone (1 per cent) in the urine.

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Dorfman, R. I., S. Schiller and W. R. Fish.: *Endocrinology* 36: 349. 1945.

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[1] Supported in part by a grant from the National Research Council Committee on research in Endocrinology.
[2] We are indebted to Ciba Pharmaceutical Products, Inc., for the generous gift of androsterone.

A SEX DIFFERENCE IN BLOOD CHOLESTEROL CONTENT IN MICE

IN THE COURSE of work on the differences in metabolism between mice of different coat colours the whole blood cholesterol has been investigated. The blood cholesterol level is known to rise with unilateral adrenalectomy, and the "normal limits" of this level in man are very wide, usually being given as 150–250 mg. per 100 cc. On the assumption that the adrenals are concerned in physiological pigmentation and the known fact that when the adrenal cortical tissue is damaged (decreased) hyperpigmentation results, the hypothesis for investigation was that mice with dark coat colour (black) should have less cortical tissue than those with light coats (agouti), and therefore might be expected to show a higher blood cholesterol content. No such differences in cholesterol between the two colours of mice could be found, but a marked difference between males and virgin females was noted. This has been investigated.

The mice used were descended from a cross made in 1944 between a male of the CBA (Strong) strain and a female of the C57 black (Little), both of these strains being pure lines. One male and three females, all agouti, resulted from this cross, and the whole of the stock has been developed from these four animals. The succeeding generations segregated for agouti and black coat colour in the ratio of 3:1. For the animals actually used in this work one male was mated to four of his litter sisters, and the blood cholesterol of the offspring determined. The hereditary factors have therefore been kept as constant as possible, bearing in mind the necessity for having mice of two different coat colours.

All the mice were kept under identical conditions of housing and diet, as has the whole colony since its origin. In every case the blood was taken when the mouse was 49 days old, this age being an arbitrary one at which the animal was sufficiently large for exsanguination to be carried out easily. Chloroform anaesthesia was used, the abdomen opened, and the blood removed from the inferior vena cava.

Cholesterol was estimated by the Liebermann-Burchard reaction, the whole blood being extracted with an ether-alcohol mixture. This method of extraction is stated to give high results when compared with the gravimetric digitonide method (Reinhold, 1936), but this is not of importance here as it is the relative amounts, not the absolute, which is the essential point. On the other hand estimation of the blue-green colour developed after 15 minutes was found to be insufficiently accurate, this stage in the reaction being too fugitive. The remarks of Snell & Snell (1937) on this point are of interest; "Practically all methods use the blue-green color, probably largely because results can be obtained so promptly." The method finally used was the estimation of the stable yellow colour developed 24 hours after the addition of the sulphuric acid, which has the additional advantage of being more immune to the action of interfering substances (Schube, 1932). The colour was estimated by an EEL photoelectric colorimeter, using a Tricolour Blue light filter. Comparison was with a natural standard developed at the same time as the solution under test. Results are regarded as accurate to within 1% and are given in the table.

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Estimation of the significance of these findings has been made by two methods. (1) By the "t" test (Fisher, 1938). This gave a value for "t" of 5.58, n being 36. This value far exceeds the limits of the published tables (Fisher & Yates, 1943), therefore by this method the sex difference is highly significant. (2) A second method was considered to be justified in view of the high degree of uniformity in housing, feeding, age and especially hereditary factors. Because of this uniformity it was considered to be legitimate to regard any male and female of these mice as constituting a "pair," in the sense used by Fisher (1938). This makes possible 357 comparisons of the data, though only 38 of these count as independent for the value of n. Estimation of significance by this method shows a mean difference between the sexes of 32.344 ± 3.991, i.e., more than eight times its standard error. The difference

WHOLE BLOOD TOTAL CHOLESTEROL IN MALE AND VIRGIN FEMALE MICE

Litter No.	Cholesterol in mg. per 100 cc.									
	Males					Virgin females				
1	200	200				163	160	149		
2	244	207	196	189	166	166				
3	183					163	160	153	153	134
4	161					146	146	135	132	
5	179	179	170			140	140			
6	164	155	149			149	149	143	143	
7	163	160				143	143			

in cholesterol content between the sexes can therefore be regarded as a real one.

Presumably this difference is due to the male requiring more cholesterol than the female. It appears very doubtful whether this can be explained on the grounds of the male needing more cholesterol than the female for the synthesis of sex hormones. In this respect it is of some interest to record that during the investigations cholesterol in five pregnant females was estimated, giving values of 211, 200, 183, 183 and 176 mg. per 100 cc. These values do not differ significantly from those found for males. It will also be noted that there was a gradual decrease in the cholesterol values found throughout the series, this being more marked in the males than in the females. (The possibility of this being due to changes in the standard solution of cholesterol has been eliminated by repeated comparisons with freshly prepared solutions). It may be associated with parental age, but no definite statement can be made on this point at this stage.

The writer has been unable to find any record of such a difference in other animals. It was at first thought that if this sex difference is also found in man the wide "normal limits" given for blood cholesterol content might be reduced to narrower separate standards for the two sexes, and the clinical usefulness of the estimation increased. However, even under the uniform conditions described the mice showed wide variations. (It should be mentioned here that none of them showed any evidence of macroscopic pathology). When the almost unlimited variations of environmental and hereditary factors in man are considered it appears very doubtful whether any immediate practical application of these findings would be possible, even if they apply to man.

SUMMARY

A sex difference in the whole blood total cholesterol content of black and agouti mice has been demonstrated, the value for males being higher than that for virgin females. This difference is statistically significant.

ACKNOWLEDGMENT

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16 KETOESTRONE IN THE KOBER REACTION

In 1931, Kober developed a colorimetric method for the determination of estrogenic steroids based upon the finding that a red color is obtained whenever estrone is heated with sulfuric acid, diluted with water, and reheated. Later Kober found the addition of phenol to the sulfuric acid before heating made for a more accurate and sensitive determination. In 1938 he found that the sensitivity of the test could be further improved by substituting 2.5% β-naphthol for the phenol sulfonic acid with other slight modifications.

Other investigators, including Cohen and Marrian (1934), Venning *et al.* (1937), Szego and Samuels (1943), and Cohen and Bates (1947), have developed various chromogenic methods for the estrogenic steroids.

Marrian (1938–39) postulated that the chromogen which is formed in the Kober reaction and which is responsible for the red color is $\Delta^{1,3,5}$ estratriene-3-ol-16, 17 dione (16 ketoestrone) as the oxonium salt. Marrian based this postulation on the following observations: a) the red colored compound was formed more easily from estriol than from estrone; b) the red color was readily discharged by mild reducing agents; c) the red colored solution deposited an insoluble precipitate when treated with o-phenylenediamine; and d) it is a well known fact that α-diketones and o-quinones yield red colored oxonium salts in acid solution.

16-Ketoestrone was not available to test the validity of Marrian's hypothesis until its synthesis was recently reported by Huffman and Lott (1948). Table 1 compares the chromogenic activity of estrone and estriol with 16-ketoestrone using the modified Kober reaction as well as other

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TABLE 1. COMPARISON OF COLOR DEVELOPMENT BY DIFFERENT PROCEDURES

	Estrone		Estriol		16-Ketoestrone	
	After 1st Heating	Final color developed	After 1st heating	Final color developed	After 1st heating	Final color development
Original Kober (1)	bright yellow	rose	yellow	pink	brownish pink	pink tinge (almost colorless)
Modified Kober (2)	blue green (green fluorescence)	orange pink	green (green fluorescence)	yellow orange	pinkish brown	very pale pink
Venning modification (4)	orange (green fluorescence)	deep pink	yellow orange (green fluorescence)	pink	rose	faint pink (almost colorless)
Cohen & Bates modification (6)	green (green fluorescence)	pale pink (green fluorescence)	yellow (green fluorescence)	pale pink (green fluorescence)	very slight brownish pink	yellow tinge (almost colorless)
Sulfuric acid (50% by vol.)	yellow orange (green fluorescence)	pale orange (green fluorescence)	pale brownish pink	colorless	light green (green fluorescence)	pale green

chromogenic procedures. Figure 1 shows the comparative color formation of estrone, estriol and 16-ketoestrone in the Kober reaction using β-naphthol.

EXPERIMENTAL PART

16-Ketoestrone was treated with the Kober reagents in the modified form (1938). The amount in solution was equilibrated with other estrogens and a study made of its absorption of light using the Sheard-Cenco spectrophotometer. The opening slit was set at $\frac{1}{2}$ mm. and the 2 mm. exit slit was used, since light intensity at 508 Mμ allowed the galvanometer to be set at 10. Previous unpublished data shows that the peak of absorption is at 508 Mμ for estrone and at 464 Mμ and 508 Mμ for estriol, using the above procedure and instruments. The concentration was 30γ per ml. The absorption

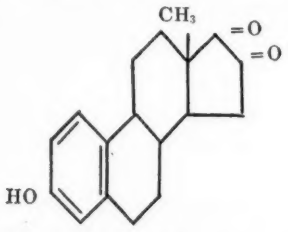

FIG. 1

was made over a range of from 400–580 Mμ. The results shown in Figure 1 is the average of several trial runs at different times on pure material using the modified Kober. All values for any wave length were within experimental error. 16-Ketoestrone was not red in color, showed very little absorption, and reached a doubtful maximal absorption at 508 Mμ or at 540 Mμ depending upon solvent used.

The samples containing 30γ per ml were obtained by diluting accurately a stock of known concentration of the pure substances. Figure 1 records the values for estrone, estriol and 16-ketoestrone when they are dissolved in 95% alcohol, and 16-ketoestrone when dissolved in ether (lower temperature can be used in removing the solvent). One ml of alcohol containing 30γ of each compound was evaporated to dryness in a hot water bath. The Kober reagent was added and heated for 2 minutes at the temperature of boiling water. The tube was then cooled, and 0.6 ml of water was added, the mixtures being kept cool. It was then reheated for $1\frac{1}{2}$ minutes and cooled for 10 seconds. Then 1.8 ml of 65% H_2SO_4 were added. The blank was 1 ml of 95% alcohol.

DISCUSSION AND CONCLUSIONS

The Kober color reaction with 16-ketoestrone was very faint and the absorption of light was found to be very small when compared to the two estrogens, estrone and estriol (equilibrated quantities). This may mean that this compound is entirely too labile to stand the heating required in the Kober reaction or that it is not chromogenic. It is therefore certainly believed that 16-ketoestrone is not responsible for the color reaction in the Kober test in the method employed in this laboratory. In fact 16-ketoestrone is comparatively colorless in all modifications of the original Kober reaction which was studied.

ACKNOWLEDGMENTS

Acknowledgment is due Parke, Davis & Company for furnishing the purified estrone and estriol used in this comparison. Further thanks is Due Dr. Max N. Huffman and Miss Mary Harriet Lott for the 16-ketoestrone.

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THE INABILITY OF THE LIVER TO INACTIVATE THE 3-METHYL ETHER OF BIS-DEHYDRODOISYNOLIC ACID[1]

The 3-methyl ether of bis-dehydrodoisynolic acid (MDDA) has been shown to have estrogenic properties (Tschopp, 1946 and others) although there is lack of agreement regarding its potency as compared with other estrogens (Nicholas, Thayer and Doisey, 1948). Segaloff (1948) has reported that MDDA, unlike most other estrogens, is not inactivated by the liver but rather is potentiated when administered through the hepatic portal system. His conclusions were based upon the finding that the intrasplenic injection of MDDA produced a greater vaginal smear response than did the subcutaneous injection of the compound. Using different criteria and methods we have examined the problem of liver inactivation of this compound under conditions of chronic administration.

MATERIALS AND METHODS

Eighteen male rats weighing approximately 80 grams were divided into three equal groups; a 26 mg. pellet of MDDA was implanted subcutaneously into each animal of the first group, in the second group similar pellets were anchored in a fold of the mesentery, and the third group was kept as normal untreated controls. Similar technique has been used extensively in showing the hepatic inactivation of steroid hormones and other substances. The criterion of effectiveness of the MDDA pellets was the depression of the growth rate which is characteristic action of estrogens in growing male rats. It was thought that growth would be inhibited less in the group having the substance absorbed through the hepatic portal system provided that the liver served as an effective inactivator of MDDA. Between the 24th and the 127th day of treatment animals were killed at intervals for the purpose of examination. At autopsy the pellets were recovered and weighed after drying; various organs were removed, weighed and preserved for histological study.

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[1] This work was aided by a grant from Ciba Pharmaceutical Products, Inc.

RESULTS

Absorption of the pellets occured at almost identical rates at the two implantation sites. Approximately half of each pellet had been absorbed by the 45th day and absorption was almost complete at 127 days.

Our results (Figure 1), using growth as a criterion of effectiveness, confirm the idea that absorption through the liver does not decrease the biological potency of MDDA. There was no indication that growth was inhibited less in animals with intramesenteric than with subcutaneous pellets. If there was any difference it was in the other direction but with the number of animals used such differences could not be considered significant. Near the

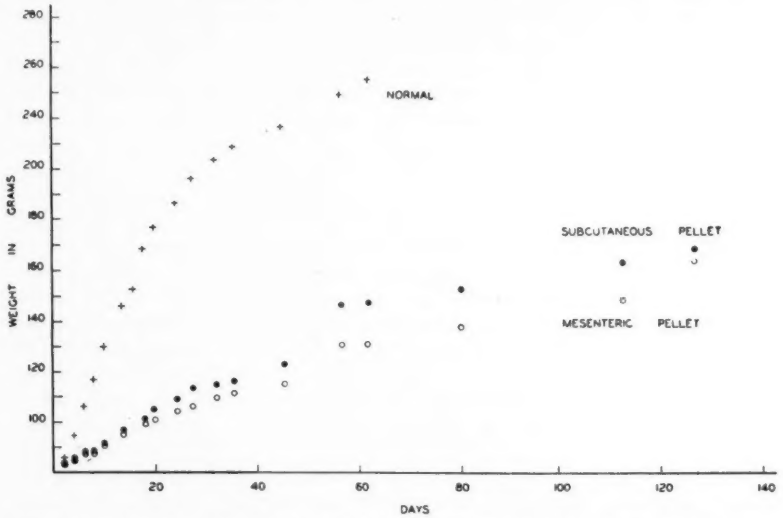

FIG. 1. Showing the mean body weight figures of male rats bearing subcutaneous pellets (solid dots) and mesenteric pellets (open circles) of MDDA.

termination of the observation period the pellets were very small and presumably the amount of MDDA absorbed daily was progressively reduced. This probably accounted for the increased growth rate toward the end of the experiment. Although the amount of MDDA being absorbed was progressively reduced the growth differential exhibited by animals with implants at the two sites was maintained, thus suggesting that dosage level was not the controlling factor.

At autopsy the adrenals and pituitaries were enlarged and the testis and seminal vesicles greatly reduced from normal size as expected from previous reports (Gaunt and Liling, 1947). There was, however, no consistent difference in the size of these organs in the animals having pellets at the two different sites. The effect of more extended chronic treatment on various organs and processes is being studied.

SUMMARY

The effectiveness of MDDA pellets in suppressing body growth was not

reduced by implantation on the mesentery as compared with a subcutaneous site. This is interpreted as a confirmation of a previous report that the liver does not inactivate MDDA.

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ASSOCIATION NOTICE

SQUIBB AND CIBA AWARDS AND AYERST, McKENNA AND HARRISON FELLOWSHIP FOR 1948. ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Association for the Study of Internal Secretions has just announced that the award provided by E. R. Squibb and Sons has been conferred on Dr. Fuller Albright of Harvard Medical School, the award furnished by the Ciba Pharmaceutical Company has been made to Dr. Carl G. Heller of the University of Oregon College of Medicine, and the Ayerst, McKenna and Harrison Fellowship has been given to Dr. Ernest M. Brown, Jr., of the George S. Cox Medical Research Institute, University of Pennsylvania.

Doctor Albright, the recipient of the Squibb Award for 1948, has contributed greatly to our understanding of a variety of endocrine functions and dysfunctions in man. His studies on hyperparathyroidism and on calcium and phosphorus metabolism have been incorporated in modern textbooks on these subjects. He has extended this work by making important additions to our knowledge of bone metabolism and its endocrine control, the formation of renal calculi and certain renal disorders. In addition he has described the syndrome of osteitis fibrosa disseminata, pigmentation and endocrine dysfunction which bears his name and he has given us one of the best concepts of Cushing's disease through investigations which applied to this condition the modern ideas of adrenal cortical physiology. Almost every field of endocrine disease has been enriched by his work. Above and beyond this he has been a lucid teacher and a constant inspiration to all his fellow students of endocrinology.

Doctor Albright was born in 1900. He received the degree of Doctor of Medicine from Harvard University in 1924. He served as House Officer at the Massachusetts General Hospital, 1924–26; as assistant medical resident at the Johns Hopkins Hospital, 1927–28 and as Moseley travelling fellow, Harvard, 1928–29. He was instructor and associate in medicine, Harvard Medical School, 1931–39; assistant professor, 1939–42; and associate professor of medicine, 1942 to the present, serving as physician to the Massachusetts General Hospital. He is past president of the American Society for Clinical Investigation and of the Association for the Study of Internal Secretions.

The Ciba Award for 1948 to Doctor Heller was given for his significant contributions to the study of the physiology of reproduction with particular reference to disorders of reproduction in man. His studies have combined techniques of the endocrine laboratory with those of the medical clinic to advance diagnostic and therapeutic procedures in various forms of hypogonadism. Doctor Heller was born in Syracuse, N. Y. January, 1913. He received the degree of Bachelor of Pharmacy in 1935 and the degree of Doctor of Philosophy and Doctor of Medicine in 1940, all from the University of Wisconsin. After interning at the Wisconsin General Hospital he held an American College of Physicians Fellowship in Medicine at Wayne University, 1941–43 and was, successively, instructor in medicine and assistant professor of physiology at Wayne University, 1943–45. Since 1945 he has been associate professor of physiology and medicine at the University of Oregon.

Doctor Ernest M. Brown, Jr. was named to receive the Ayerst, McKenna and Harrison Fellowship for 1948. He was born in 1919 and received the degrees of Bachelor of Arts from West Virginia University in 1941 and of Doctor of Medicine from the University of Pennsylvania in 1944. He served as intern and junior resident at the University of Pennsylvania Hospital 1944–46. Since that time until April 1948 he has been a member of the Army Medical Corps. Doctor Brown will work at the George S. Cox Medical Research Institute with Dr. F. D. W. Lukens on lesions of the islands of Langerhans produced by intravascular infusion of glucose.

AUTHOR INDEX, VOLUME 42

ASTWOOD, E. B., see STANLEY, M. M., 107

BENNETT, L. L., see GORDAN, G. S., 153

BIRNBERG, C. H., see SHERBER, D. A., 20

BIRNIE, J. H., W. J. EVERSOLE AND R. GAUNT. The extra-renal action of desoxycorticosterone: Survival and water intoxication studies, 412

BIRNIE, J. H., see EVERSOLE, W. J., 482

BORELL, U., AND HJ. HOLMGREN. The effect of methylthiouracil on the oxygen consumption of the thyroid, liver and kidneys, 427

BRIGGS, G. M., see HAQUE, M. E., 273

BROWNELL, K. A., AND F. A. HARTMAN. Increased glyconeogenetic factor production after adrenal enucleation, 232

BRUZZONE, S., see LIPSCHUTZ, A., 201

CAVETT, J. W., see MCCLENDON, J. F., 168

CHAIKOFF, I. L., see ENTENMAN, C., 210, 215

CHAIKOFF, I. L., see WOLFF, J., 468

CHANUTIN, A., see LUDEWIG, S., 352

CHARIPPER, H. A., see D'ANGELO, S. A., 399

CHOH HAO LI, see GORDAN, G. S., 153

D'ANGELO, S. A., A. S. GORDON AND H. A. CHARIPPER. The effect of inanition on the anterior pituitary-adrenocortical interrelationship in the guinea pig, 399

DAVIS, M. E., AND EDITH L. POTTER. The response of the human fetal reproductive system to the administration of diethylstilbestrol and testosterone propionate during early pregnancy, 370

DELSON, B., S. LUBIN AND S. R. M. REYNOLDS. Spiral arteries in the human ovary, 124

DEMPSEY, E. W., Book Review. Textbook of Endocrinology, by Hans Selye, 424

DEMPSEY, E. W., see WISLOCKI, G. B., 56

DESBARATS, M. L., see HOFFMAN, M. M., 279

DIEKE, SALLY H., The effect of removing various endocrine glands on the hair cycles of black rats, 315

DOHAN, F. C., AND F. D. W. LUKENS. Experimental diabetes produced by the administration of glucose, 244

DORFMAN, R. I. Studies on the bioassay of hormones: The assay of testosterone propionate and androsterone by a chick comb inunction method, 1

DORFMAN, R. I. Studies on the bioassay of hormones: The relative reactivity of the comb of various breeds of chicks to androgens, 7

DORFMAN, R. I., AND ADELINE S. DORFMAN. Studies on the bioassay of hormones—The assay of estrogens by a chick oviduct method, 85

DORFMAN, R. I., AND ADELINE S. DORFMAN. Studies on the bioassay of hormones—The comparative oviduct response of various breeds of chicks to stilbestrol, 102

DORFMAN, R. I., ADELINE S. DORFMAN AND DOROTHA GRIM. Stud-

ies on the bioassay of hormones —The assay of pregnant mare's serum chorionic gonadotrophin, 93

Dorfman, R. I., Adeline S. Dorfman and Dorotha Grim. Studies on the bioassay of hormones —The comparative activity of estrogens by the oviduct response in chicks, 98

Dorfman, R. I., Ethelreda Ross and R. A. Shipley. Metabolism of the steroid hormones—The Metabolism of progesterone and ethynyl testosterone, 77

Dorfman, R. I., Jane E. Wise and R. A. Shipley. Metabolism of the steroid hormones—The conversion of isoandrosterone to androsterone, 81

Dorfman, Adeline S., see Dorfman, R. I., 85

Dorfman, Adeline S., see Dorfman, R. I., 93

Dorfman, Adeline S., see Dorfman, R. I., 98

Dorfman, Adeline S., see Dorfman, R. I., 102

Dorfman, R. I., see Miller, A. M., 174

Dorfman, R. I., see Schiller, Sara, 476

Dunphy, J. E., Book Review. George Crile, An Autobiography, Edited, with Sidelights, by Grace Crile, 329

Dury, A., and E. D. Robin. Urethane-induced lymphopenia in normal and adrenalectomized rats, 320

Entenman, C., I. L. Chaikoff, T. Gillman and F. L. Reichert. Choline prevents fatty change and cirrhosis in the livers of dogs subjected to hypophysectomy and thyroidectomy, 215

Entenman, C., I. L. Chaikoff and F. L. Reichert. The influence of hypophysectomy, thyroidectomy, and of both hypophysectomy and thyroidectomy upon the fat content of the liver of the dog, 210

Evans, H. M., see Gordan, G. S., 153

Everett, J. W., see Miller, D. C., 421

Eversole, W. J., J. H. Birnie and R. Gaunt. The inability of the liver to inactivate the 3-methyl ether of bis-dehydrodoisynolic acid, 482

Eversole, W. J., see Birnie, J. H., 412

Fawcett, D. W. Histological observations on the relation of insulin to the deposition of glycogen in adipose tissue, 454

Foster, W. C., see McClendon, J. F., 168

Friedman, N. B. The pathology of the adrenal gland in Addison's disease with special reference to adrenocortical contraction, 181

Furth, J., see Sobel, H., 436

Gabrio, B. W., and K. Salomon. Calcium deposition in the femora of chick embryos after the injection of estradiol benzoate, 73

Gaunt, R., see Birnie, J. H., 412

Gaunt, R., see Eversole, W. J., 482

Gillman, T., see Entenman, C., 215

Gordan, G. S., L. L. Bennett, Choh Hao Li and H. M. Evans. The effect of dietary protein content upon the nitrogen retention and weight gain produced by the hypophyseal growth hormone, 153

Gordon, A. S., see D'Angelo, S. A., 399

Grauer, R. C., W. F. Starkey and Eleanor Saier. The influence of mixtures of androsterone

and dehydroisoandrosterone on biological response, 141
GRIM, DOROTHA, see DORFMAN, R. I., 93, 98

HAQUE, M. E., R. J. LILLIE, C. S. SHAFFNER AND G. M. BRIGGS. Effect of vitamin deficiencies in New Hampshire Chicks injected with high doses of thyroxine, 273
HARTMAN, F. A., see BROWNELL, K. A., 232
HECHTER, O. Lymphocyte discharge from the isolated rabbit spleen by adrenal cortical extract, 285
HECHTER, O., see STONE, D., 307
HELLER, V. G., AND R. H. THAYER. Chemical changes in the blood composition of chickens and turkeys fed synthetic estrogens, 161
HILL, R. T. Grafted mouse ovaries and their adrenal cortical function, 339
HOFFMAN, M. M., G. MASSON AND M. L. DESBARATS. The role of bile in the absorption of steroid hormones from the gastro-intestinal tract, 279
HOFFMANN, E., see WHEELER, R. S., 326
HOLMGREN, HJ., see BORELL, U., 427
HUMEREZ, J., see LIPSCHUTZ, A., 201
HUTCHENS, T. T., see MIKKELSEN, W. P., 394

IGLESIAS, R., see LIPSCHUTZ, A., 201

KURZROK, R., see LEONARD, S. L., 176
KURZROK, R., see PERLMAN, P. L., 26
KURZROK, R., see SHERBER, D. A., 20

LEA, A. J. A sex difference in blood cholesterol content in mice, 477
LEATHEM, J. H., AND R. D. SEELEY. Plasma and liver protein concentrations of hypothyroid rats, 150
LEONARD, S. L., P. L. PERLMAN AND R. KURZROK. Hyaluronidase levels of rat testes as related to age, hypophysectomy and cryptorchidism, 176
LEONARD, S. L., see PERLMAN, P. L., 26
LILLIE, R. J., see HAQUE, M. E., 273
LIPSCHUTZ, A., R. IGLESIAS, S. BRUZZONE, J. HUMEREZ AND J. M. PEÑARANDA. Progesterone and desoxycorticosterone in the steroid control of the gonadotrophic function of the hypophysis, 201
LUBIN, S., see DELSON, B., 124
LUDEWIG, S., AND A. CHANUTIN. Cholesterol studies of adrenals and plasma in partially hepatectomized and partially nephrectomized rats after injection of HN3, 352
LUKENS, F. D. W., see DOHAN, F. C., 244

MARLOW, H. W. 16 ketosterone in the kober reaction, 479
MASSON, G., see HOFFMAN, M. M., 279
MCCLENDON, J. F., W. C. FOSTER AND J. W. CAVETT. An attempt to explain the anomalous action of Lugol's solution in exophthalmic goiter, 168
MIKKELSEN, W. P., AND T. T. HUTCHENS. Lymphopenia following electrically induced convulsions in male psychotic patients, 394
MILLER, A. M., AND R. I. DORFMAN. Metabolism of the steroid hormones: The isolation of etiocholandediol-3 (α), 17 (α) from the human urine, 174
MILLER, D. C., AND J. W. EVERETT. Ascorbic acid concentration and cholesterol storage in the corpus

luteum of the pregnant rat under experimental conditions, 421
Moore, D. H. Effect of reciprocal steroid treatment on the electrophoretic patterns of fowl sera, 38
Moya, F., J. L. Prado, R. Rodriguez, K. Savard and H. Selye. Effect of the dietary protein concentration upon the secretion of adrenocorticotrophin, 223

Parker, F., Jr., see Robbins, S. L., 237
Peñaranda, J. M., see Lipschutz, A., 201
Perlman, P. L., S. L. Leonard and R. Kurzrok. Some factors influencing the liberation of hyaluronidase from testes homogenate and spermatozoa in the rat, 26
Perlman, P. L., see Leonard, S. L., 176
Persike, E. C. Increased protein catabolism in thyroidectomized rat: Anatomical changes, 363
Persike, E. C. Increased protein catabolism in thyroidectomized rats: Rates of urine urea excretion and serum urea concentrations, 356
Potter, Edith L., see Davis, M. E., 370
Prado, L., see Moya, F., 223
Pugsley, L. I., and S. Rampton. Variables affecting the assay of insulin, 31

Quimby, F. H. Organ weights of rats receiving hormone supplements during recovery from chronic starvation, 263

Rampton, S., see Pugsley, L. I., 31
Reichert, F. L., see Entenman, C., 210, 215
Reynolds, S. R. M., see Delson, B., 124
Robbins, S. L., and F. Parker, Jr. The use of the male North American frog (rana pipiens) in the diagnosis of pregnancy, 237
Robin, E. D., see Dury, A., 320
Rogers, P. V., and C. P. Richter. Anatomical comparison between the adrenal glands of wild Norway, wild alexandrine and domestic Norway rats, 46
Richter, C. P., see Rogers, P. V., 46
Rodriguez, R., see Moya, F., 223
Ross, Ethelreda, see Dorfman, R. I., 77

Saier, Eleanor, see Grauer, R. C., 141
Salomon, K., see Gabrio, B. W., 73
Savard, K., see Moya, F., 223
Sayers, G., see Sayers, Marion A., 379
Sayers, Marion A., G. Sayers and L. A. Woodbury. The assay of adrenocorticotrophic hormone by the adrenal ascorbic acid-depletion method, 379
Schiller, Sara, and R. I. Dorfman. Metabolism of the steroid hormones: Androsterone administration to guinea pigs, 476
Seeley, R. D., see Leathem, J. H., 150
Segaloff, A. The effect of partial hepatectomy on the *in vivo* activation of triphenylchloroethylene, 472
Segaloff, A. The metabolism of some chemical degradation products of estrogens. Westerfeld's lactone, bis-dehydro-doisynolic acid, estrololactone and β-estradiol, 15
Selye, H., see Moya, F., 223
Shaffner, C. S., see Haque, M. E., 273
Sherber, D. A., C. H. Birnberg and R. Kurzrok. Viscosimetric determination of the hyaluronidase content of spermatozoa, 20

SHIPLEY, R. A., see DORFMAN, R. I., 77

SHIPLEY, R. A., see DORFMAN, R. I., 81

SOBEL, H., AND J. FURTH. Hypervolemia in mice bearing granulosa cell growths: Time of onset and some associated physiological and chemical changes, 436

STANLEY, M. M., AND E. B. ASTWOOD. The accumulation of radioactive iodide by the thyroid gland in normal and thyrotoxic subjects and the effect of thiocyanate on its discharge, 107

STARKEY, W. F., see GRAUER, R. C., 141

STONE, D., AND O. HECHTER. Splenic lymphocyte discharge induced by adrenal cortical hormones under *in vivo* conditions, 307

STRICKLER, H. S. Urinary steroid excretion patterns of two pseudohermaphrodites, 230

THAYER, R. H., see HELLER, V. G., 161

TRENTIN, J. J., AND C. W. TURNER. Effect of ultraviolet irradiation on biological activity of α-estradiol benzoate, 448

TURNER, C. W., see TRENTIN, J. J., 448

WHEELER, R. S., AND E. HOFFMANN. Goiterous chicks from thyroprotein-fed hens, 326

WISE, JANE E., see DORFMAN, R. I., 81

WISLOCKI, G. B., AND E. W. DEMPSEY. The chemical histology and cytology of the pineal body and neurohypophysis, 56

WOLFF, J., AND I. L. CHAIKOFF. Plasma inorganic iodide, a chemical regulator of normal thyroid function, 468

WOODBURY, L. A., see SAYERS, M. A., 379

ZARROW, M. X. The role of the steroid hormones in the relaxation of the symphysis pubis of the guinea pig, 129

SUBJECT INDEX, VOLUME 42

Addison's disease
  pathology of adrenal in, 181
Adrenals
  of different species of rat, 46
  pathology of Addison's disease, 181
  glycogenetic factor of, 232
  and lymphocyte discharge from spleen, 285, 315
  effect of urethane on lymphopenia, 320
  simulation by ovary, 339
  stimulation by nitrogen mustards, 352
  ascorbic acid of, 379
  effect of inanition on, 399
Age
  and response to androgens, 7
  and hyaluronidase content of testis, 176
Androgen
  assay of, 1, 7
  effect of electrophoretic patterns of blood, 38
  metabolism of, 77, 81, 174, 476
  effects of mixtures of, 141
  prenatal effects of, 370
Antithyroid agents
  effect of thiocyanate on thyroid, 107
  and oxygen consumption of organs, 427
Ascorbic acid
  of adrenal cortex, 379
  of corpus luteum, 421
Assay
  of androgens, 1, 7
  of insulin, 31, 448
  of estrogens, 85, 98
  of chorionic gonadotropin, 93, 237
  of adrenocorticotrophin, 379
Association Notice, 331, 485

Blood
  electrophoretic patterns and steroids, 38
  plasma proteins in hypothyroidism, 150
  composition affected by estrogens, 161
  plasma cholesterol after nitrogen mustards, 352
  volume after granulosal tumors 436
  cholesterol in sexes, 477
Blood vessels
  of human ovary, 124

Calcium metabolism
  effects of estrogens on, 73
Chick comb assay for androgen, 1, 7
Cryptorchidism
  and hyaluronidase content of testis, 176

Desoxycorticosterone
  as pituitary regulator, 201
  extra-renal action of, 412
Diabetes
  produced by glucose, 244
Diet
  effect of growth hormone response, 153
  and secretion of adrenocorticotrophin, 223
  starvation and organ weights, 263
  inanition and adrenal, 399

End-organ responsiveness
  and age, 7
  in different breeds, 102
  in vitamin deficiencies, 273
Enzymes
  hyaluronidase of spermatozoa, 20
  hyaluronidase of spermatozoa, 26
  hyaluronidase of testis, 176
Estrogens
  metabolism of, 15
  effect of electrophoretic pattern of blood, 38
  and calcification, 73

assay of, 85, 98
effect on oviduct, 102
effect on symphysis pubis, 129
and blood composition, 161
prenatal effects of, 370
effect of ultraviolet light on, 448
metabolism in liver, 472
color reactions of, 479

Fat
metabolism of liver, 210, 215
relation to glycogen, 454

Goiter
effect of iodine on, 168
in chicks, 326
Gonadotrophin
assay of pregnant mare serum, 93
effect of steroids upon, 201
pregnancy test with male frog, 237
Growth hormone
effect of diet on effect of, 153

Hair
growth and endocrine glands, 315
Hermaphroditism
steroid secretion in, 230
prenatal effects of steroids, 370
Histochemistry
of pineal and neurohypophysis, 56
of fat and glycogen, 454

Insulin
assay of, 31
relation to fat, 454
Iodine
effect of thiocyanate on metabolism of, 107
effect on goiter, 168
as regulator of thyroid, 468

Kidney
oxygen consumption after methylthiouracil, 427

Liver
protein in hypothyroidism, 150
fat metabolism of, 210, 215
role of bile in absorption of hormones, 279
oxygen consumption after methylthiouracil, 427
and estrogen metabolism, 472
and bisdehydrodoisynolic acid, 482
Lymphocytes
discharge from spleen, 285, 315
effect of urethane upon, 320
and electro-shock, 394

New Books
George Crile, An Autobiography, 329
Textbook of Endocrinology, 424

Ovary
spiral arteries in, 124
adrenal cortical function of, 339
ascorbic acid in, 421
blood volume and granulosal tumors, 436
Oviduct
and assay of estrogens, 85, 98
responsiveness in different breeds, 102

Pancreas
diabetes produced by glucose, 244
Pineal gland
chemical histology and cytology of, 56
Pituitary gland
chemical histology and cytology of neurohypophysis, 56
growth hormone of, 153
gonadotrophin regulation, 201
effect on liver fat, 210, 215
adrenocorticotrophin regulation of, 223
adrenocorticotrophin content of, 379
inanition and adrenocorticotrophin, 399
Pregnancy
test with male frogs, 237
Progesterone
metabolism of, 77
effect on symphysis pubis, 129
effect on pituitary gonadotrophin, 201

Radioactive iodine
accumulation in thyrotoxicosis, 107
Reproductive system
effects of prenatal estrogens and androgens, 370

Spermatozoa
hyaluronidase content of, 20, 26
Symphysis pubis
relaxation of by steroid hormones, 129

Testis
hyaluronidase of, 176
Thiocyanate
effect of iodine metabolism, 107
Thyroid
effect of thiocyanate on, 107
effect of iodine on, 168
effect on liver fat, 210, 215
effect on protein catabolism, 356, 363
oxygen consumption after methylthiouracil, 427
regulation by iodide, 468
Thyroxine
and vitamin deficiencies, 273